Demonstration of a RNA-dependent nuclear interaction between the promyelocytic leukaemia protein and glyceraldehyde-3-phosphate dehydrogenase

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The promyelocytic leukaemia (protein) (PML) localizes to multi-protein complexes known as PML nuclear bodies. We found that glyceraldehyde-3-phosphate dehydrogenase (GAPDH) co-immunoprecipitates with PML and co-localizes with PML in nuclear bodies. RNase treatment disrupts the ability of PML and GAPDH to both co-localize and co-immunoprecipitate, indi-

cating that the association between PML and GAPDH depends on the presence of RNA. Disruption of PML bodies contributes towards reduced apoptosis in acute promyelocytic leukaemia and GAPDH induces apoptotic neuronal death. The GAPDH–PML interaction may be involved in the regulation of apoptosis.

INTRODUCTION

In acute promyelocytic leukaemia (APL), myeloid differentiation is blocked, leading to an accumulation of promyelocytes (for reviews, see [1,2]). In APL, the promyelocytic leukaemia protein (PML) is fused to the retinoic acid receptor α (RAR α) as a result of a chromosomal translocation [1,2]. Overexpression of the PML–RAR α fusion protein in mice induces pathological changes that are similar to those found in human APL [3]. PML seems to be present in all tissues and cell lines [1,2]. In normal cells, the majority of PML is localized to large multiprotein complexes in the nucleus. These nuclear bodies are distinct from other nuclear organelles, such as spliceosomes and nucleoli [1,2]. There are, on average, 10–30 nuclear bodies per cell; however, both the morphology and number of these change during the cell cycle [1,2,4]. In APL cells, PML no longer localizes to nuclear bodies [1,2].

PML is a member of the RING zinc-finger family of proteins. There are over 80 members of this distinct zinc-finger family [5,6]. PML contains two cysteine-rich zinc-binding domains, referred to as the B1 B-box and B2 B-box respectively, which are followed by a leucine coiled-coil domain [2,5,6]. The RING, B1 B-box, B2 B-box and leucine coiled-coil domains are collectively referred to as the tripartite domain, or the RBCC motif. The RBCC motif is found in several other proteins involved in oncogenesis (for a review, see [5]). All three of these domains appear to be important in the mediation of protein–protein interactions [6–8].

PML's role in growth control and apoptosis depends on its subcellular localization. PML suppresses transformation of fibroblasts by the *neu* oncogene [1,2,9]. PML overexpression in fibroblasts leads to increased levels of apoptosis during serum starvation [10]. Mutations in the RBCC region of PML result in a diffuse nuclear localization without discernible nuclear bodies [7,11]. Inability to form nuclear bodies has been associated with

a loss of growth and suppression of transformation activity in PML [8]. Mutational analysis indicates that PML's pro-apoptotic action is mediated through its RING domain [10]. Other experiments have indicated that the inducible expression of PML–RAR α is apoptotic, and support the view that the RING domain is required for pro-apoptosis [12]. The subcellular localization of PML is modified by different types of viral infection. The single-stranded RNA arenaviruses redistribute PML to the cytoplasm [11a]. The lessened commitment to apoptosis observed in arenavirus-infected cells may depend on the translocation of PML from the nucleus [10].

We have sought to elucidate the protein–protein interactions of PML as a means of understanding its roles in normal and pathological cell function. Other workers have shown that the glycolytic enzyme and pro-apoptotic protein, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), can show a punctate nuclear localization [13], which seemed to us to be similar to that observed for PML. Accordingly, we used co-immunoprecipitation and double-labelling immunocytochemistry with confocal laser microscopy for mouse and human fibroblasts to determine whether there is evidence for an interaction between the two proteins. We found that PML and GAPDH co-immunoprecipitate and that they co-localize in a subset of nuclear bodies. The localization of PML and GAPDH to the same nuclear bodies is dependent on the presence of RNA.

MATERIALS AND METHODS

Cell culture

NIH 3T3 (ATCC CRL-1658) cells and the human fibroblasts 551 (ATCC CCL-110) were grown and maintained in 10% (v/v) fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (Gibco). In serum-withdrawal experiments, cells were grown until they were 50–70% confluent, washed three times in serum-free media, and then fresh serum-free media was added.

Abbreviations used: APL, acute promyelocytic leukaemia; DMP, dimethyl pimelimidate; eIF-4E, elongation initiation factor 4E; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mAb, monoclonal antibody; PML, promyelocytic leukaemia (protein); RAR α , retinoic acid receptor α ; RT, room temperature; NDP, nuclear dot protein.

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Control cells were washed, but fresh media with serum was added.

PML antibodies

The PML antibodies used were either a monoclonal antibody (mAb) to the human form of PML, mAb 5E10 [4], or a polyclonal antibody which reacts with both human and mouse forms of PML [7,14]. This antibody was used to detect PML in transgenic mice experiments [15].

Co-immunoprecipitation studies of GAPDH and PML

GAPDH mAb (Chemicon, Temecula, CA, U.S.A.) or mAb5E10 was covalently attached to Protein A-Sepharose (Pharmacia, Piscataway, NJ, U.S.A.) by cross-linking with dimethyl pimelimidate (DMP) using standard procedures described previously [16,17]. Briefly, Protein A-Sepharose beads were washed twice in 0.2 M sodium borate, pH 9.0. For every 1 ml of beads, 2 mg of antibody/ml was used. These were mixed continuously for 2 h at room temperature (RT). Beads were washed with sodium borate solution three times, followed by a wash with 0.2 M triethanolamine, pH 8.5. An equal volume of 40 mM DMP in 0.2 M triethanolamine was then added. The mixture was gently agitated for 1 h at RT. The mixture was spun and supernatant removed. An equal volume of 0.2 M ethanolamine, pH 8.2, was added to the beads for 5 min at RT. Beads were washed with the sodium borate solution, and stored at 4 °C until use. Beads were rinsed in PBS before use.

Cells were washed twice in serum-free media, trypsin-treated, transferred to a Falcon tube with media and sera and pelleted at 800 *g* at RT for 5 min. Pellets were washed three times in media prewarmed to 37 °C. Cells were lysed in buffer IPB [150 mM NaCl/20 mM Tris/HCl (pH 7.4)/1% Nonidet P40/100 μ M PMSF/5 μ g/ml of the following protease inhibitors: chemo-statins, leupeptin, pepstatin A, aprotinin and antipain]. Cells were spun at 800–1000 *g* for 5 min at 4 °C. Pellets were resuspended and spun at 13000 *g* for 20 min at 4 °C. Preclearing was then carried out by the method of Harlow and Lane [18]. Supernatants were collected, and 20 μ l of Protein A-Sepharose plus mouse Ig was added. Beads were mixed for at least 1 h at 4 °C. Samples were spun in order to remove beads. Protein A-GAPDH beads were added to the supernatant and mixed for 2 h at 4 °C. Beads were then washed three times with IPB, and an additional three times with modified IPB [containing 0.1% (v/v) deoxycholate and no Nonidet P40]. Beads were spun and collected. An equal volume of reducing sample buffer was added in preparation for SDS/PAGE.

Gel electrophoresis and Western blot analysis

SDS/PAGE (12% gels) was carried out using standard procedures. Gels were blotted on to Immobilon P (Millipore, Bedford, MA, U.S.A.) and probed with antibodies using standard techniques, as described previously [18]. Western blot analysis was carried out using the electrochemiluminescence (ECL) method (Amersham, Arlington, IL, U.S.A.) to detect bound antibodies. The total protein concentration of each sample was determined such that equal amounts of protein were loaded on to gels. Protein assays were carried out using bicinchoninic acid protein assay system (Pierce, Rockford, IL, U.S.A.).

Immunolocalization studies

NIH 3T3 cells were grown overnight on coverslips in 10% (v/v) FBS/Dulbecco's modified Eagle's medium. Cells were fixed in

methanol for 10 min at –20 °C. Immunofluorescence was carried out as described previously [7,11]. GAPDH mouse mAb (Chemicon) was used at 1:500 dilution, and the PML rabbit polyclonal antibody at 1:200. The PML antibody was a gift of K. Howe and E. Solomon (Guy's Hospital, London, U.K.) and P. Freemont (Imperial Cancer Research Fund, London, U.K.) and has been characterized extensively [7,14]. Goat anti-rabbit fluorescein isothiocyanate (FITC) and donkey anti-mouse Texas Red secondary antibodies were used at dilutions of 1:200 and 1:100 respectively. Images were collected on either a Zeiss or Leica confocal microscope with minimal pinhole settings, in order to obtain minimal *z*-axis thickness of the optical sections (minimal *z*-axis thickness increases the probability that co-localized immunoreaction is in the same plane).

FITC was excited at 488 nm, and emission filters restricted detector activation to 523–537 nm. Texas Red was excited at 568 nm, and emission greater than 590 nm was allowed to reach the detectors. The FITC and Texas Red fluorescences were recorded sequentially for identical image fields to avoid cross-talk between the channels. Care was taken to ensure that there was no breakthrough of the FITC signal into the Texas Red channel, or vice versa.

The number of cells studied in each sample exceeded 500 and each experiment was repeated at least twice. Grey scale confocal images were coloured red or green and digitally added using Northern exposure software (Empix Ltd., Mississauga, Ont., Canada). For the purposes of counting nuclear bodies, the images were enlarged in Photoshop (Adobe, San José, CA, U.S.A.).

RNase treatment of coverslips

Cells fixed on coverslips were treated with DNase-free RNase or buffer. Coverslips were washed twice in PBS, followed by two washes with 2 $\times$ SSC (where 1 $\times$ SSC is 0.15 M NaCl/0.015 M sodium citrate). Coverslips were incubated at 37 °C for 30 min with either 100 μ g of RNase/ml or buffer only. At the end of the treatment cells were washed twice with 2 $\times$ SSC and once with PBS. Coverslips were then stained as described above. RNase (Boehringer Mannheim, Indianapolis, IN, U.S.A.) was prepared as described by Sambrook et al. [19], where RNase was boiled in order to eliminate any residual DNase.

RNase treatment of lysates

Human fibroblast 551 cells were harvested as described above. Before immunoprecipitation with the antibody of interest, pre-cleared total cell lysates were treated with RNase for 30 min at 37 °C with either 20 μ g of RNase/ml or buffer only. At the end of this treatment, cells were immunoprecipitated as described above.

RESULTS

GAPDH and PML interact in NIH 3T3 and human fibroblasts

Previous reports [13] showed that GAPDH has a punctate nuclear distribution, together with a dense uniform distribution in the cytosol of HeLa cells. We found that GAPDH has a similar nuclear distribution in NIH 3T3 cells and human 551 fibroblasts, and that the punctate nuclear distribution resembled that found for PML nuclear bodies (see [4,11], and references therein). Double immunoreaction with GAPDH mAb (Figure 1A1) and a PML polyclonal antibody (Figure 1A2) revealed a similar nuclear localization for the two proteins. The PML polyclonal antibody has been well characterized, and has been shown to recognize both mouse and human PMLs [7,14,15]. In

Figure 1 Co-localization of GAPDH and PML in NIH 3T3 cells

Cells were immunoreacted for GAPDH, as shown by the red-coloured image (A1), for PML, as shown by the green-coloured image (A2), or digitally added image, where yellow/orange indicates co-localization (A3). The images show typical PML–GAPDH co-localization in a subset of nuclear bodies. In RNase-treated cells, the number of GAPDH-immunoreactive nuclear bodies decrease (B2) and GAPDH–PML co-localization disappeared (B3). All confocal settings were identical for the top and bottom panels.

Figure 2 GAPDH and PML co-immunoprecipitate

(A) NIH 3T3 lysates were co-immunoprecipitated with the GAPDH mouse mAb. A Western blot probed with the PML antibody is shown. PML is approx. 69 kDa with higher molecular-mass isoforms; lower bands on the gel represent degradation products. Samples in the lower panels were probed with eIF-4E (shown by band at 25 kDa). (B) Samples were co-immunoprecipitated with mAb 5E10. Below, aliquots of the same samples were treated as above, and the subsequent blots were probed with the trk antibody (Santa Cruz); trk is 140 kDa. + serum and — serum are as described in the Results section; Lysate, total cell lysate; S, supernatant from the co-immunoprecipitation step; preclear, beads from the preclear step (see the Materials and methods section); co-IP, immunoprecipitated fraction; W, wash.

these and other images, we scanned identical fields sequentially for the immunoreaction of the two proteins. Different excitation and emission wavelengths were utilized on the sequential scans and control images were taken to ensure that any co-localization did not represent fluorescent bleed-through. The grey scale confocal images were recoloured red (GAPDH immunoreaction) or green (PML immunoreaction), and were digitally added so that orange/yellow colouration revealed regions in which the immunoreaction was co-localized (Figure 1A3). The extent of co-localization was $57 \pm 18\%$ (means $\pm$ S.D.) of PML bodies with GAPDH and $37 \pm 14\%$ of GAPDH bodies with PML. The total number of bodies counted was 332. As shown in Figure 1(A3), much of the co-localization was found in a distinct subset of nuclear bodies. Preliminary observations suggest that cells with low passage numbers have greater numbers of nuclear GAPDH bodies and, consequently, a higher proportion with GAPDH–PML co-localization (results not shown).

Our co-immunoprecipitation studies showed either that there is a direct binding between PML and GAPDH, or that the two proteins bind to common elements in NIH 3T3 cells. Lysates were prepared from cells, which were washed with media to remove serum, and thereby serum-deplete the cells (–serum in Figure 2). Other cells were similarly washed, but serum was reintroduced (+serum in Figure 2). Cells were harvested after 3, 6, 9 and 12 h after washing. In the first experiments, the GAPDH mAb was used for co-immunoprecipitation and the resulting Western blot was probed using the PML antibody (see Figure 2A). These blots showed that PML and GAPDH associated under both serum deprivation and normal serum conditions. The level of association did not vary as a function of time after washing for the normal serum condition. The amount of PML present in the co-immune precipitation was slightly reduced at times greater than 9 h in the serum-withdrawal condition. PML

was present in the supernatant lane, indicating that not all of the PML and GAPDH were associated, which appeared to be consistent with the co-localization of only a subset of nuclear bodies shown in Figure 1. PML has several isoforms, ranging in size from 47 to 100 kDa [20]. The 69 kDa isoform was most prevalent in our studies, but higher-molecular-mass isoforms were observed as well, together with degraded PML fragments.

The ability of GAPDH to co-immunoprecipitate with other proteins was assayed as a negative control. As shown in the lower panel of Figure 2(A), Western blots demonstrated that GAPDH did not co-immunoprecipitate the elongation-initiation factor 4E (eIF-4E), another protein that has a punctate nuclear distribution [21]. There was no eIF-4E present in any of the co-immunoprecipitation lanes, despite being present in the total lysate, supernatant and preclear lanes at the expected molecular mass of 25 kDa. Another nuclear protein, Jun, also did not co-immunoprecipitate (results not shown).

To complement the immunoprecipitation and immunolocalization studies obtained with the polyclonal PML, experiments were conducted with mAb 5E10, an mAb characterized as being specific for human PML [4]. mAb 5E10 was used to immunoprecipitate whole-cell extracts from the human fibroblast cells 551. Experiments were carried out similarly to those described above. Figure 2(B) shows that the mAb 5E10 precipitated with GAPDH at 6 h, but not 12 h after washing, in both conditions. The presence of co-immunoprecipitation at 12 h in the experiments illustrated in Figure 2(A) and the absence at 12 h in the experiments illustrated in Figure 2(B) may be due to time-dependent masking of the mAb 5E10 epitope. PML can undergo post-translational modification, including phosphorylation [9] and attachment of a ubiquitin-like protein [14]. Either modification could alter accessibility of other proteins to the mAb 5E10 epitope site.

Figure 3 PML and GAPDH do not immunoprecipitate in RNase-treated cells

Human fibroblast 551 cells were immunoprecipitated with either the mAb 5E10 (PML IP) or the GAPDH mAb (GAPDH IP). The blot was probed with the mAb against GAPDH. IP, immunoprecipitation; lysate, total cell lysate; pre-clear and pellet are as described in the Materials and methods section.

Intriguingly, when NIH 3T3 cells were transfected with human PML, GAPDH did not co-immunoprecipitate with the exogenous protein (results not shown). This shows that some portion of exogenous PML or its associated elements is either absent or interferes with the PML–GAPDH interaction that was found for endogenous PML.

RNase may mediate the PML–GAPDH interaction

RNase treatment was found to eliminate the co-localization found for a subset of nuclear bodies. NIH 3T3 cells on coverslips were treated with either DNase-free RNase or buffer. Figures 1(B1), 1(B2) and 1(B3) show typical images for RNase-treated cells. The numbers and intensity of nuclear GAPDH bodies decreased dramatically after RNase treatment (compare Figures 1B1 and 1A1). The numbers and intensity of PML nuclear bodies seemed unaffected by RNase treatment (compare Figures 1B2 and 1A2), similar to previous reports [4].

PML and GAPDH do not co-immunoprecipitate in RNase-treated cells

In order to confirm the immunofluorescence studies showing the loss of GAPDH and PML association in RNase-treated cells (Figure 1), cell lysates were treated with RNase and immunoprecipitated (Figure 3). RNase treatment was performed as described for the corresponding immunofluorescence experiments. Cells were lysed before RNase treatment. Control cell lysates received buffer only and were incubated in tandem with the treated cells. Human fibroblast 551 cells were co-immunoprecipitated with the PML mAb 5E10, and the subsequent Western blots were probed with a GAPDH mAb. As expected, GAPDH appears in the total cell lysate (lysate) at the appropriate size (37 kDa). As a positive control, cells were immunoprecipitated with the GAPDH antibody (GAPDH IP). Our data indicate that the GAPDH antibody precipitates GAPDH, as expected. Cells immunoprecipitated with the PML antibody (PML IP), but not treated with RNase, immunoprecipitated GAPDH, as shown in Figure 2. However, in RNase-treated cell lysates, GAPDH is not immunoprecipitated by PML (Figure 3).

DISCUSSION

GAPDH may contribute towards the pathogenesis of certain neurodegenerative diseases by binding to mutated proteins that have been found in familial forms of the diseases. These diseases

include Huntington's disease, dentatorubralpallidoluysian atrophy, Kennedy's disease, Machado-Joseph and spinocerebellar ataxia type 1 (for reviews, see [22,23]). At the molecular level, GAPDH possesses several activities unassociated with its glycolytic function, including a uracil DNA-glycosylase activity, microtubule binding and RNA binding, specifically AU-rich RNA and tRNA [22]. GAPDH binds non-translated regions of hepatitis RNA and hammerhead ribozymes [24–26]. GAPDH can discriminate between wild-type and mutant tRNAs that are defective in nuclear–cytoplasmic transport [13]. A portion of GAPDH is found in the polysomal fraction under certain conditions [24], which might position it for a role in RNA transport. The above associations have led to the suggestion that GAPDH is an RNA chaperone [22].

GAPDH plays a role in apoptosis. GAPDH levels increase, and it densely translocates to the nucleus during the early stages of apoptosis [27,28] in cells, including HEK293, PC12, S49 and primary thymocytes [27]. Both mRNA and protein levels of GAPDH increase in cultured cerebellar and cerebrocortical neurons undergoing age-induced apoptosis [28]. Addition of antisense oligonucleotides to GAPDH decreased apoptosis caused by cytosine arabinucleoside in the cerebellar and cerebrocortical neurons [27,28].

Implications of the PML–GAPDH interaction for PML function

PML nuclear bodies have been shown consistently to contain specific proteins, including PML, SP100, nuclear dot protein (NDP) 55, and NDP52 [2]. Other proteins associate with a subset of PML nuclear bodies, including P0, P1, P2, retinoblastoma protein and the ret finger protein Rfp [2,11a,14,30]. GAPDH appears to fall into the latter category. It remains to be determined whether or not GAPDH associates with PML in the presence of specific subsets of the above proteins. PML associates with another RNA binding protein, P0 [11a], a ribosomal protein that has both a nuclear and cytoplasmic localization ([11a], and references therein). Our preliminary data indicate that GAPDH binds P0 in co-immunoprecipitation assays (results not shown). PML bodies are dynamic, and PML shuttles between the nucleus and cytoplasm [31]. Nuclease treatment does not disrupt the structure of PML nuclear bodies [4]; however, our data indicate that RNA forms an essential functional component necessary for the association of PML and GAPDH in at least some of the bodies. Recently, RNA has been found inside a subset of the nuclear bodies, but it did not seem to be associated directly with PML [32]. Furthermore, the RNase experiments suggest that GAPDH would only associate with those nuclear bodies that contain RNA. If GAPDH does serve as a RNA chaperone, then PML nuclear bodies may be involved in that activity.

Transfected PML does not associate with endogenous GAPDH, supporting the premise that there are functional components not present in exogenous PML bodies. Consistently with this, GAPDH neither co-immunoprecipitates nor co-localizes (K. L. B. Borden, unpublished work) with exogenous bodies. Transfection of PML is unlikely to lead to up-regulation of other PML body components, including both protein and nucleic acid components.

Implications for APL

The effect of the presence of the PML–RAR α fusion protein found in cells of APL patients on the PML–GAPDH association is not clear. Interestingly, both co-immunoprecipitation and co-localization studies in the APL cell line NB4 showed that this interaction was either absent or present only at very low levels (results not shown). However, there was a punctate nuclear

component of GAPDH present in these cells. Treatment of NB4 cells with retinoic acid did not cause reassociation of GAPDH with PML nuclear bodies. This might indicate that, although PML-associated nuclear bodies are reformed during retinoic acid treatment, not all other components reassociate with the nuclear bodies, e.g. another PML nuclear-body component, Rfp, only associated with some nuclear bodies in NB4 cells, and levels of association did not increase greatly when cells were treated with retinoic acid, whereas, in non-transformed cell lines, the PML–Rfp interaction is normally observed in a subset of bodies [30]. These conclusions suggest that NB4 cells treated with retinoic acid may not provide a good system for the study of normal PML body interactions. We cannot rule out the possibility that the PML–GAPDH interaction may be cell-type-specific.

The role that PML–GAPDH association plays in neurodegenerative conditions is not yet defined. GAPDH is purported to bind Huntingtin, the protein responsible for Huntington's disease [23]. In particular, it appears that GAPDH binds to polyglutamine repeats found in several proteins associated with neurodegenerative conditions. In these diseases, severity is directly correlated with the number of polyglutamine repeats observed. However, the data reported thus far indicate that GAPDH may bind these proteins indirectly, and it is unknown which subcellular population of GAPDH is involved in these associations and what the intermediary proteins may be. Alternatively, it has been suggested that the neurodegenerative action of GAPDH is a result of overexpression of GAPDH and subsequent accumulation of GAPDH in the nucleus, which may trigger apoptotic events [27,33]. Thus the interplay between GAPDH and PML may be crucial to apoptosis. APL is associated with a decrease in apoptosis, while neurodegenerative diseases feature increased apoptosis [34]. Given the established pro-apoptotic activities of PML and GAPDH, variations in the PML–GAPDH nuclear interaction may have the capacity to either promote or retard apoptosis, and thereby have a corresponding capacity to foster diseases involving either decreased or increased apoptosis.

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