Surface residue mutations of the PML RING finger domain alter the formation of nuclear matrix-associated PML bodies

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SUMMARY

The human protein PML, was first identified as part of a fusion protein with retinoic acid receptor alpha as found in the chromosomal translocation which gives rise to acute promyelocytic leukaemia. PML is normally localised to large matrix-associated nuclear domains (known as ND10, Kr bodies, PODs or PML NBs) which comprise several multi-protein complexes. Within the PML protein, there are a number of identified zinc-binding domains, one of which called the RING finger is found in a large family of diverse and unrelated proteins. Here, we report the effect of site-directed mutations within the context of the whole PML protein, of amino acids found on the surface of the PML RING finger domain and PML NB formation in vivo. Mutations of a small region of the RING finger domain surface affect the size and numbers of PML NBs in a mouse fibroblast expression assay, resulting in fewer but larger exogenous PML NBs. Mutations of other surface RING residues, however, do not affect exogenous PML NB formation. Furthermore, all of the PML RING mutants co-localise to both endogenous and exogenous wild-type PML NBs. These data identify a specific region of the PML RING finger domain which is directly involved in correct PML NB formation. They also provide evidence to suggest that the PML RING finger is involved in mediating PML-PML oligomeric interactions, as part of a mechanism leading to the assembly of the PML NB complex.

Key words : APL, PML, RING finger, Mutagenesis, Nuclear body

INTRODUCTION

PML nuclear bodies are nuclear multi-protein complexes which are distinct from other sub-nuclear structures (Nickerson et al., 1995). They were first identified by using monoclonal autoantibodies, human autoimmune antisera and antibodies raised against rat liver matrix depleted in lamins (Ascoli and Maul, 1991; Stuurman et al., 1992), and were later shown to contain the PML proto-oncoprotein (Dyck et al., 1994; Koken et al., 1994; Weis et al., 1994). These multi-protein structures are known as ND10, Kr bodies, PML oncogenic domains or PODs or PML nuclear bodies (PML NBs; for review see Doucas and Evans, 1996). There are about 10-30 PML NBs per cell and they range in diameter from about 0.2-0.3 μm, although the number and morphology of these structures varies considerably throughout the cell cycle (Ascoli and Maul, 1991; Stuurman et al., 1992). PML NBs do not appear to be associated with chromatin, but are tightly bound to the nuclear matrix (Chang et al., 1995; M. N. Boddy and P. Freemont, unpublished results) and contain several proteins identified mainly through screening autoimmune antisera for immunofluorescence patterns that overlap with that of the PML NBs (Ascoli and Maul, 1991; Boddy et al., 1996; for review see Doucas and Evans, 1996).

The role of PML NBs is still unknown and the importance of their integrity for PML function is not fully understood. However, an accumulating body of data shows that PML NBs are the target for a variety of virus-derived proteins and that subsequent loss of organisation of these domains is an important phase of the viral infection cycle (Maul and Everett, 1994; Puvion-Dutilleul, et al., 1995; Carvalho et al., 1995; Doucas et al., 1996; Ishov and Maul, 1996; Szekely et al., 1996). Furthermore, in acute promyelocytic leukemia (APL), a PML-retinoic acid receptor alpha fusion protein results in a block in promyelocyte differentiation and a disruption of wild-type PML NBs (for review see Grimwade and Solomon, 1997). Interestingly, upon treatment with all-trans retinoic acid APL cells differentiate. This is associated with a reformation of wild-type PML NBs, implying that the integrity of PML NBs appears important in normal promyelocyte differentiation (Daniel et al., 1993; Dyck et al., 1994; Weis et al., 1994; Koken et al., 1994), although mutants of PML-RARA which do not delocalise PML NBs are still functionally active in blocking differentiation (Grignani et al., 1996).

The PML protein comprises a number of characterised zinc-binding domains which includes a RING finger (Borden et al., 1995a; for review see Saurin et al., 1996) adjacent to two cysteine/histidine rich motifs known as B1 and B2 boxes (Reddy et al., 1992; Borden et al., 1995b). These domains together with a predicted α-helical coiled-coil domain form a conserved motif (known as RBCC) which is found in several other unrelated proto-oncoproteins (Reddy et al., 1992; see Saurin et al., 1996 for review). The PML RING finger appears...
to be required for PML NB, formation as deletion of the RING and/or point mutations of the zinc binding ligands, abrogate PML NB formation in vivo (Kastner et al., 1992; Borden et al., 1995a; Le et al., 1996). Recently, we solved the three-dimensional solution structure of the PML RING finger domain (Borden et al., 1995a; Borden and Freemont, 1996). Here, we extend these studies by describing the effects, in vivo, of a number of site-directed mutations of the RING domain, on the formation of PML NBs, using a mouse fibroblast expression assay. We show that several amino acid mutations clustered on part of the surface of the RING finger, which would not affect the structural integrity of the domain, dramatically alters the number and size of exogenous PML NBs. These data show that the PML RING finger domain is important in mediating PML homo-oligomeric interactions which appear essential for the assembly, size and number of PML NBs in vivo.

MATERIALS AND METHODS

Modelling and electrostatic surface potential calculations of the PML RING finger

Amino acid mutations of the PML RING finger structure were modelled by using the coordinates from the NMR solution structure (Borden et al., 1995a; PDB entry 1BOR) and the program ‘O’ (Jones et al., 1991). Mutated RING structures were visualised on a Silicon Graphics Indigo2 workstation using the program ‘PREPI’ (S. Islam and M. Sternberg, ICRF). Electrostatic surface potential calculations were carried out using the program ‘GRASP’ (Nicholls et al., 1991) with standard default parameters and visualised on an Silicon Graphics Indigo2 workstation.

Site-directed mutations of PML

Mutagenesis of the PML RING domain was performed using the PCR ‘stitching’ method of Mullis et al. (1986). PML(69 kDa)-MLVPLINK (20 ng) was used as a template for all PCR reactions. Primers were used at 100 ng each per reaction. PCR cycling conditions were the same for all mutations. A proofreading polymerase, Pfu (Promega) was used and all clones were sequenced to confirm the mutations and fidelity of remaining sequences. The PML RING finger was mutated as follows (numbering refers to Fig. 1C). A PML ‘casette’, bases 15-419 was used for PCR amplification with the following ‘master’ primer sequences (forward: 5’ CTG GGG TCC ATG GAG CCT GCA CCC GCC GC 3’; reverse: 5’ CGA CTA TTT GGA GCG GGT GC 3’). Two separate PCR reactions were performed, using the ‘master’ forward primer and reverse mutant primer (see below), and the ‘master’ reverse primer and forward mutant primer (see below). Each PCR product was gel purified and used in the final PCR reaction together with the original ‘master’ primers. This amplifies a fragment (15-419 bp) carrying the desired mutations, which after digestion with NcoI and AarII was ligated into the MLVPLINK vector. The following ‘internal’ mutant primers (forward and reverse) were used to make the specific mutations.

PML Cys9,12 Ala: 5’ CAG TTT CTT CGC GCC CAG CAA GCC CAG GCG 3’; 5’ CGC CTG GCC TGG CTT GGC GGC GAG CAA CTT G 3’;
PML Cys40,43 Ala: 5’ GCC ATG CAG GCA CCC ATC GCA CAG GCC GC 3’; 5’ CGC CTG TGG GAT GGC TGC CTG CAT GCC 3’;
PML Glu1(3) AlaGlu Arg: 5’ GAG CCC CGG CTT CGG CGG AGT TCC AGT TTC TGC GC 3’;

5’ GCG CAG AAA CTG GAA CGG CTC CGC CGA AGC GGG GGC TC 3’;
PML Glu39ΔGlu: 5’ GCC ATG GAG TGC CCC ATC TGC C 3’;
5’ GCC AGA TGG GGC ACT CCA TGC C 3’;
PML Glu44ΔGlu: 5’ CCC ATC TGC GAG GGC CCC TGG 3’;
5’ CCA GGG CGC TCT GCA GAT GGG 3’;
PML Glu(10,11,13) ΔGlu+Arg8ΔSer: 5’ CCA GTT TCT GAG CTG CGA GGA ATG CGA GGC G 3’;
5’ GCC TCT GCA TTT CTC GCA GCT CAA CTG AAA G 3’;
PML Arg8ΔGlu: 5’ CCA GTT TCT GAG CTG CGA GGA ATG CC 3’;
5’ GCC ATT GCT GGC ACT CCA GAA ACT G 3’;
PML Glu15ΔArg: 5’ CCA GGC GGC AGC CAA GTG CC 3’;
5’ GCC ACT TGG CTC GCG CCT G 3’;
PML Lys17ΔGlu: 5’ CGG AAC CGG AGT GCC CGA AGC 3’;
5’ GCT TCG GCC ACT CGG CTT CCG 3’;
PML Lys17,Glu15ΔAla: 5’ GCC AGG CGG CAG CGG CCT GCC CGA AGC 3’;
5’ GCT TCG GCC AGC CGG CTG CCT GCC C 3’;
PML Lys17,20 Ala: 5’ GCC GGA AGC CGC GTG GCC CTC GCT GCT GCC TTG 3’;
5’ CAA GGC AGC AGC GCC GAC CAC GCG GCT TCC GCC G 3’.

Transient transfection; immunofluorescence staining and western blotting

NIH3T3 cells were transiently transfected using the DEAE dextran shock method with 8 µg of each construct. Both wild-type PML and PML mutants were transfected in a mammalian expression vector carrying the MLV enhancer (Dalton and Treisman, 1992). In addition to wild-type PML, PML was additionally N-terminally tagged with the myc epitope recognised by the 9E10 monoclonal antibody. For immunofluorescence studies cells were fixed 24 or 40 hours after transfection at −20°C in methanol for 10 minutes. Transfected PML protein was detected using a variety of different antibodies depending on the experiment and/or expression construct. Neat affinity purified anti-PML polyclonal antiserum that does not detect endogenous mouse PML was used, followed by an anti-rabbit FITC conjugated secondary antibody at 1:200 dilution (v/v) (Dakopatts Ltd). The PML antiserum was also used crude recognising both transfected and endogenous mouse PML. Transfected wild-type PML and PML mutants were also detected using either the 5E10 monoclonal antibody, that has been shown to detect PML (1:20 v/v; Stuurman et al., 1992; Koken et al., 1994), or the myc 9E10 antibody, with an anti-mouse Texas Red-conjugated secondary antibody (Amersham). For western blot analysis, total protein extracts (20 µg) were separated on 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel, and blotted onto nitrocellulose membranes (Amersham). Membranes were blocked with 3% fat-free milk in PBS, pH 7.6, 137 mM NaCl, then incubated with different antibodies, depending on the experiment, in PBS for 1 hour at room temperature. Subsequently, membranes were incubated with an anti-mouse peroxidase-conjugated secondary antibody at 1:2,000 dilution (v/v) (Amersham). Each of the incubation steps were followed for 3 washes for 10 minutes in PBS, 0.1% Tween-20. Labelling was performed as described in the ECL protocol (Amersham).

Measurements and counting of PML NBs in vivo

Slides were scanned on a Bio-Rad MRC 1000 confocal microscope (microscope settings were constant for all experiments) in a systematic fashion, although the number of transfected cells counted was
RESULTS

Modelling and electrostatic surface potential of the PML RING finger domain

We have previously solved the three-dimensional structure of the PML RING finger domain in solution (Borden et al., 1995a). The structure revealed that the PML RING finger is a compact domain, folded around two zinc atoms, which displays some distinctive surface features (Borden et al., 1995a). In this study, we used this structure to design several site-directed mutations which we based on a number of criteria. One over-riding consideration, was that mutations were chosen on the basis that they would not affect the tertiary structure and, therefore, the overall fold of the PML RING domain. This would allow us to probe primarily the surface features of the RING finger, including potential molecular interaction sites, without destabilising the overall fold of the domain. One striking feature of the PML RING finger, is the large surface area of positive electrostatic potential, surrounded by discrete smaller negatively charged areas (Fig. 1A). This suggests that the RING surface could be important in mediating molecular interactions perhaps through complimentary electrostatic interactions. We therefore, altered both the electrostatic surface potential as well as the nature of the molecular surface itself.

After visual inspection of the PML RING finger structure, surface residues were either chosen individually or as groups of residues for mutagenesis studies (Fig. 1B and C). All the mutations were modelled onto the RING finger structural framework and analysed for possible perturbations to the overall RING domain fold. This was done by visual inspection, which took into account the proximity of the mutations to amino acids directly involved in either the zinc-dependent folding of the domain (Cys/His zinc ligands) or formation of the hydrophobic core. None of the mutations appeared to alter the structural core of the domain, although it is not possible to exclude any effects that might occur on the RING domain folding process in vivo. However, as shown in Fig. 1, several mutations were isosteric and therefore considered to be subtle as they changed the charge of the residue, without altering the sidechain length (for example Gln to Glu). Other chosen mutations were direct charge inversions, that is Lys (+) to Glu (−) or Glu (−) to Arg (+). Alanine mutations were also made such that the role of individual residues could be assessed (Fig. 1).

Transient transfection of wild-type PML and PML mutants in mouse fibroblasts

The PML (69 kDa) RING finger mutants as shown in Fig. 1B, were made and cloned into the MLV-PLINK mammalian...
expression vector (Dalton and Treisman, 1992). Mutations were confirmed by sequencing and equivalent amounts of each PML mutant construct and a wild-type PML (69 kDa) control were transfected into NIH3T3 mouse fibroblasts cells. Transfected cells were cultured for 48 hours and then subjected to indirect immunofluorescence with either the human PML affinity purified polyclonal (Borden et al., 1995a) or the 5E10 PML monoclonal antibody (Stuurman et al., 1992).

The wild-type PML (69 kDa) control gave the characteristic punctate nuclear labelling (Fig. 2A) observed in several previous transfection experiments (Kastner et al., 1992; Borden et al., 1995a,b, 1996). The following PML (69 kDa) mutants also produced the characteristic wild-type staining pattern: Glu(1-3)ΔAla,Glu,Arg, Gln39ΔGlu, Gln44ΔGlu, Gln(10,11,13) ΔGlu + Arg8ΔSer and Arg8ΔGlu. Representative examples of the labelling observed for Gln(10,11,13)ΔGlu+Arg8ΔSer and Arg8ΔGlu. The following PML (69 kDa) mutants also produced the characteristic wild-type staining pattern: Glu(1-3)ΔAla,Glu,Arg, Gln39ΔGlu, Gln44ΔGlu, Gln(10,11,13) ΔGlu + Arg8ΔSer and Arg8ΔGlu. Representative examples of the labelling observed for Gln(10,11,13)ΔGlu+Arg8ΔSer and Arg8ΔGlu. The following PML (69 kDa) mutants also produced the characteristic wild-type staining pattern: Glu(1-3)ΔAla,Glu,Arg, Gln39ΔGlu, Gln44ΔGlu, Gln(10,11,13) ΔGlu + Arg8ΔSer and Arg8ΔGlu. Representative examples of the labelling observed for Gln(10,11,13)ΔGlu+Arg8ΔSer and Arg8ΔGlu. The following PML (69 kDa) mutants also produced the characteristic wild-type staining pattern: Glu(1-3)ΔAla,Glu,Arg, Gln39ΔGlu, Gln44ΔGlu, Gln(10,11,13) ΔGlu + Arg8ΔSer and Arg8ΔGlu. Representative examples of the labelling observed for Gln(10,11,13)ΔGlu+Arg8ΔSer and Arg8ΔGlu. The following PML (69 kDa) mutants also produced the characteristic wild-type staining pattern: Glu(1-3)ΔAla,Glu,Arg, Gln39ΔGlu, Gln44ΔGlu, Gln(10,11,13) ΔGlu + Arg8ΔSer and Arg8ΔGlu. Representative examples of the labelling observed for Gln(10,11,13)ΔGlu+Arg8ΔSer and Arg8ΔGlu. The following PML (69 kDa) mutants also produced the characteristic wild-type staining pattern: Glu(1-3)ΔAla,Glu,Arg, Gln39ΔGlu, Gln44ΔGlu, Gln(10,11,13) ΔGlu + Arg8ΔSer and Arg8ΔGlu. Representative examples of the labelling observed for Gln(10,11,13)ΔGlu+Arg8ΔSer and Arg8ΔGlu.

However, the PML RING mutants Lys17ΔGlu (Fig. 3C), Glu15ΔArg (Fig. 3D), Lys17,Glu15ΔAla (Fig. 3E) and Lys17Lys20ΔAla (Fig. 3F) displayed aberrant labelling patterns with the appearance of only a few PML NBs per cell (~2 to 4), which were considerably larger on average, than transfected wild-type PML (69 kDa) NB structures. As can be seen in Fig. 3, each of the mutants had fewer but larger transfected PML NBs when compared to the wild-type controls (compare Fig. 3B with C,D,E,F). Also in Fig. 3 is a view of the altered electrostatic surface potential, compared to wild type, resulting from the amino acid mutation/s which give rise to the abnormal PML NB phenotype. One notable feature which was common in all of the transfected mutant PML NB experiments was the appearance of mutant NBs coalescing as illustrated in Fig. 3G. Although, this phenomenon could sometimes been seen in 3T3 cells expressing high amounts of wild-type PML, it was particularly common for the mutant transfections possibly contributing to the observable phenotype of small numbers of larger PML NBs.

All of the above observations were reproducible over five subsequent repeat experiments, using differing batches of cells and transfection protocols, suggesting that the phenotype was specific and due only to the amino acid mutations within the RING finger domain. However, in order to obtain a more quantitative interpretation of these results, the PML (69 kDa) mutants Glu15ΔArg, Lys17ΔGlu, Glu15,Lys17ΔAla, Lys17Lys20ΔAla and wild-type PML (69 kDa) were again transfected into NIH3T3 cells, using equivalent amounts of plasmid DNA, in three separate experiments. Measurements of both the number and diameter size of the observed PML NBs were undertaken for each transfection experiment (see Materials and Methods). In order to eliminate the possibility that the observed mutant phenotype was due to differential levels of protein expression, western blot analysis was carried out on PML wild type and two different mutants (Fig. 4). The results from these experiments were consistent for both wild type and mutants. A representative graph of the distribution of PML NB sizes for the mutants and wild-type PML (69 kDa) is shown in Fig. 5 with standard deviations from several experiments listed in Table 1A. In all experiments the distributions for the number of PML NBs per cell for each of the mutants, as represented as a frequency of the total number of measurements, was tight with >80% of the observations ranging between 1 and 4 PML NBs per cell (Fig. 5A; Table 1A).
However, the distribution for wild-type PML was broader with the average number of PML NBs per cell considerably higher varying between 7 and 11 with larger standard deviations (Fig. 5A; Table 1A). The average diameter of wild-type PML NBs fluoresces is 1.4 μm, whereas mutant PML NBs are of considerably larger average diameter of 1.8 to 2.5 μm (Fig. 5B; Table 1A). Although the standard deviations of the observed diameter sizes were relatively large, reflecting the distribution of sizes, it is clear from Fig. 5B that the mutants were consistently larger than wild type. Furthermore, it appears that the distribution of sizes for mutant NBs appear to form discrete sub-populations of NB diameter size (Fig. 5B). It was found that 20 to 40% percent of the mutants in all the experiments have a similar diameter size to wild type with 10% of wild type having more mutant-like PML NBs (Fig. 5B). However, for a fixed transfection time, between 60 and 80% of the total observations for each of the mutants showed larger PML NBs than wild type (Fig. 5B).

In summary, for a transient experiment of fixed time, PML wild-type forms on average 9.3 exogenous NBs per cell of average diameter size 1.4 μm compared to some of the PML RING mutants, which on average form 2.4 to 3.8 exogenous NBs per cell of average diameter size 1.8 to 2.5 μm. These observations are also independent of protein expression levels, as shown by western blots of transfected cells.

**Co-transfection of wild-type PML and PML Lys17→Glu in mouse cells.**

In order to test whether the mutant phenotype, as observed in the transient expression studies in mouse cells, was affected...
by co-expression of wild-type PML, a co-transfection study was carried out. Tagged PML Lys17ΔGlu and untagged PML wild type were transiently co-transfected in NIH3T3 cells, and the resulting PML NB distribution analysed (data not shown). After analysing a large number of transfected cells, the mutant phenotype of small numbers of large PML NBs, was not apparent in cells co-expressing both mutant and wild-type PML, although we consistently observed smaller numbers of mutant NBs per cell of wild-type size (data not shown).

### Transient transfection of wild-type PML and PML mutants in human cells

In order to test the properties of the PML mutants in human cells, HeLa cells were transiently transfected with Myc-tagged wild-type PML and PML Lys17ΔGlu and Lys17.Lys20ΔAla mutants in three separate experiments using two different transfection times (20 and 48 hours). The expressed proteins were visualised by immunofluorescence using the 9E10 myc monoclonal antibody, with endogenous PML localised using the PML polyclonal antibody. By using separate wavelength channels, it was possible to detect only the Texas Red (myc-tag) immunofluorescence thereby allowing us to see whether transfected wild-type PML and mutants co-localise to sites of endogenous PML.

As can be seen in Fig. 6, both wild-type PML (6Ai) and the Lys17ΔGlu (6Aii) mutant clearly co-localise to sites of endogenous PML as illustrated by a complete superposition of the myc-tagged PML immunofluorescence signal with the signal from that of the PML polyclonal which recognises both transfected and endogenous PML. Furthermore, co-transfections of PML wild type with PML Lys17ΔGlu showed complete co-localisation between both PML proteins (data not shown).

Further studies were undertaken to test whether the PML NB phenotype, as seen for transient expression of RING mutants in mouse cells, was observed in human cells. Myc-tagged PML Lys17ΔGlu and wild type were transfected separately into HeLa cells and localised using immunofluorescence with the 9E10 Myc monoclonal antibody, allowing only transfected PML to be visualised. Western blot analysis was carried out for mutant and
that endogenous and transfected PML co-localise. (Ai) Transfected PML wild type. (Aii) Transfected PML Lys17ΔGlu. The very small PML speckles observed in (i) and (ii) were not included in the final NB counting and measurement analyses (see Materials and Methods). (B) Expression levels of wild-type PML and Myc-tagged PML Lys17ΔGlu proteins. A Coomassie Blue-stained gel in the left panel is shown to qualitate the concentration of samples loaded on the immunoblot shown in the right panel. Transfected myc-tagged PML was detected using the Myc monoclonal antibody 9E10. (1) Untransfected cells, (2) wild-type PML (3) PML Lys17ΔGlu. Protein size markers (in kDa) are indicated in the middle of the two panels. The asterisk shows a faint band corresponding to endogenous Myc protein.

wild type to check for equivalent transfected protein expression (Fig. 6B). The transfected PML NBs for both wild type and mutant were counted from two separate experiments in several cells and their apparent diameters measured, the results of which are shown in Table 1B. Both experiments were reproducible, and it was clear that the PML RING mutant and wild type had very similar distributions of sizes as seen in Table 1B where the mean diameter sizes of wild type and Lys17ΔGlu mutant are very similar. However, the mean number of transfected PML NBs per cell for mutant (5.0±1.9) as compared to wild type (9.0±2.1) was lower which was also reflected in the relevant distributions (data not shown). Interestingly, the average number and mean diameter size of wild-type PML NBs transfected in either HeLa cells or NIH3T3 cells are very similar (Table 1). In summary, the results from the transfection studies in human cells showed that the mutant PML NB phenotype as observed in mouse cells is not readily observable although there appears to be consistently less mutant NBs per cell as compared to wild type.

Co-localisation of the PML NB component PIC1 with mutant PML NBs

In order to test whether other PML NB components could associate with mutant PML NBs, we studied the co-localisation of a recently identified PML NB component called PIC1 (Boddy et al., 1996). Co-transfections in both mouse (NIH3T3) and human cells (HeLa and HT1080 cells) of PIC1 and PML Lys17,Lys20ΔAla RING mutant were carried out. In both cases PIC1 co-localised to the mutant transfected PML NBs, showing that the mutant NBs could incorporate another PML NB component (Fig. 7). Furthermore, using an antibody
specific to PIC1, we observed endogenous PIC1 co-localising both to transfected PML mutant and wild-type NBs (data not shown). Interestingly, co-transfections of PIC1 with the other PML RING mutants showed in all cases co-localisation of PIC1 with mutant PML (data not shown).

DISCUSSION

Delineation of a specific region of the PML RING finger required for normal PML nuclear body formation

We first described the three-dimensional structure of the PML RING domain and showed that mutations of the zinc binding ligands which were necessary for the structural integrity of the domain, prevented PML NB formation in transfected cells (Borden et al., 1995a). Others have also shown that the RING finger domain is necessary for PML NB formation in transfected cells, in that either mutations or deletion of the domain prevents NB formation (Kastner et al., 1994; Le et al., 1996). Furthermore, point mutations and/or deletion of either the B1-B2 B-box domains or α-helical coiled coil domain also prevented PML NB formation and that the RING and B1, B2 B-boxes were not necessary for PML homo-oligomerisation (Borden et al., 1996; Le et al., 1996). The conclusions from these studies suggests that the RBCC domain acts as an integral structural domain which is involved in mediating the inter-molecular interactions necessary for PML NB formation in vivo and that PML homo-oligomerisation is mediated by the helical coiled-coil domain. It is interesting that the ability to form PML NBs is directly correlated with the ability of PML to suppress growth and transformation in a mouse fibroblast transformation assay (Le et al., 1996). In this study, we found that mutations of amino acids Glu15, Lys17 and Lys20 (Fig. 1) which localise to a specific region of the RING finger domain could affect the shape and morphology of PML NBs in transfected mouse cells, without disrupting them. Furthermore, we also observed that combined mutations of other surface residues in other regions had little or no effect on PML NB formation, showing that the integrity of this particular surface area was important for the formation of PML NBs. However, the exact contribution to the observed NB phenotype of the individual mutated amino acids is unclear, given that changing either the nature of the electrostatic surface potential in this area from either positive/negative to all negative (Lys17ΔGlu) or all positive (Glu15ΔArg) or to neutral (Lys17, Lys20ΔAla) resulted in the same PML NB phenotype (Fig. 3). However, from our studies it is clear that a small surface area of the PML RING finger is important in mediating the molecular interactions which are necessary for correct PML NB formation and that mutations in this area will affect this process in some way.

Abnormal PML nuclear body formation appears suppressed by the co-expression of wild-type PML

From our transfection studies, the apparent phenotype of large PML NBs, is not readily observable for PML RING mutants expressed in human cells, although we did observe smaller number of exogenous NBs (Fig. 5; Table 1). This is also true for co-transfection experiments of RING mutants and PML wild type, in both human and mouse cells, where both wild type and mutant PML proteins also co-localise to sites of endogenous mouse or human PML (Fig. 6). An obvious explanation for these results, is that the mutant phenotype is not observed in the presence of either endogenous or exogenous wild-type PML. However, the numbers and size of wild-type exogenous PML NBs in both mouse and human cells are very similar (Table 1), showing that exogenous PML can form similar sized NBs, irrespective of cell type (mouse versus human). In HeLa cells, we consistently observed nearly half as many exogenous NBs in cells expressing the PML RING mutant as compared to wild type, similar to our observations in mouse cells (Table 1). It is plausible that abnormally large mutant PML NBs can only be observed in stably transfected human cell lines expressing the PML RING mutants, an area requiring further study.

Mechanistic implications for the formation of nuclear bodies by PML

In order to test whether the association of the PML RING mutants with other known PML NB components is altered in some way, we studied the association of the recently described PML interacting protein PIC1 (Boddy et al., 1996) with the RING mutants. From these experiments, it was clear that both endogenous mouse and human PIC1, as well as exogenous human PIC1 could localise and associate with mutant PML NBs similar to wild-type NBs (Fig. 7). This suggests that for at least one known PML NB component, the mutant NB phenotype is not due to a disruption of an interaction between PML and PIC1. However, PML NBs are known to contain at least six other protein components (Doucas and Evans, 1996) and each component would have to be studied in this light.

It has been observed that transfected exogenous PML NBs primarily contain only PML, although small non-stoichiometric amounts of known endogenous PML NB components can be detected in these exogenous structures (G. Maul, personal communication). The PML NB transfection assay used in this study is, therefore, probably testing PML-PML oligomeric interactions. We have identified a region of the PML RING finger domain which appears important in mediating these interactions, since mutations in this region allows PML NBs to become abnormally large. One trivial explanation for our results is that the PML point mutants aggregate non-specifically. However, this seems unlikely since: (1) other multiple-mutations of the RING surface had no effect. (2) mutations which unfold the RING domain form a diffuse ‘aggregation’ pattern. (3) there is an equilibrium between discrete populations of large and small NBs in the mutants. Indeed, small numbers of wild-type PML NBs are of similar size to the PML mutant (<10%; Fig. 5B), with a significant percentage of mutant PML NBs similar to wild-type NBs (20-40%; Fig. 5B). The ability of the mutants to affect protein-protein associations may simply alter the distribution between small and large NBs, such that mutant PML NBs can more easily coalesce than wild type, thereby forming larger structures (see Fig. 3G). This mechanism is partially supported by the observation that discrete sub-populations of PML NB diameter sizes are observed (see Fig. 5B), which could only result from the fusing or coalescence of discrete NB sizes assuming that a sphere adequately represents the observed NBs.

In summary, we have identified a region on the molecular
surface of the PML RING finger domain which is important in
mediating exogenous PML NB formation in mouse fibroblasts.
These data strongly suggest that PML-PML oligomeric inter-
actions are important for the correct assembly of PML NBs and
that surface mutations of the RING domain can result in an
increased prevalence of PML NB coalescence or fusion.

We thank Kathy Howe and Ellen Solomon for comments and
Richard Newman for help and assistance in counting and measuring
the PML NBs. We are grateful for the use of the 5E10 monoclonal
antibody reported by Stuurman et al. (1992) and the 9E10 monoclonal
antibody (G. Evan). This work was supported by the ICRF and by the
EEC in the form of a TMR fellowship to E.D.

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(Received 25 March 1997 – Accepted 10 July 1997)