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FAST TRACKS

Biochemical Fractionation Reveals Association of DNA Methyltransferase (Dnmt) 3b With Dnmt1 and That of Dnmt 3a With a Histone H3 Methyltransferase and Hdac1

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Abstract De novo DNA methyltransferases, Dnmt3a and 3b, were purified by fractionation of S-100 extract from mouse lymphosarcoma cells through several chromatographic matrices followed by glycerol density gradient centrifugation. Dnmt3a was separated from Dnmt3b and Dnmt1 in the first column, Q-Sepharose whereas Dnmt3b co-purified with Dnmt1 after further fractionation through Mono-S and Mono-Q columns and glycerol density gradient centrifugation. Following purification, the majority of de novo DNA methyltransfearse activity was associated with Dnmt3b/Dnmt1 fractions. By contrast, the fractions containing Dnmt3a alone exhibited markedly reduced activity, which correlated with diminished expression of this isoform in these cells. Histone deacetylase 1(Hdac1) cofractionated with Dnmt3a throughout purification whereas Hdac1 was separated from Dnmt3b/Dnmt1 following chromatography on Mono-Q column. Dnmt3a purified through glycerol gradient centrifugation was also associated with a histone H3 methyltransferase (HMTase) activity whereas purified Dnmt3b/Dnmt1 was devoid of any HMTase activity. The activity of this HMTase was abolished when lysine 9 of N-terminal histone H3 peptide was replaced by leucine whereas mutation of lysine 4 to leucine inhibited this activity only partially. This is the first report on the identification of a few key co-repressors associated with endogenous Dnmt3a and of a complex containing Dnmt3b and a minor form of Dnmt1 following extensive biochemical fractionation. J. Cell. Biochem. 88: 855–864, 2003.

Key words: DNA methylation; Dnmt3a; Dnmt3b; Dnmt1; Hdac1; histone methyltransferase

Methylation at 5-position of cytosines of complementary CpG base pairs in DNA is an epigenetic process evolved in vertebrates. Alteration of the methylation profile of some genes is known to cause developmental abnormalities or various diseased conditions [Bird and Wolffe, 1999; Bestor, 2000; Robertson and Wolffe, 2000a]. Three different families of DNA (cytosine-5) methyltransferase Dnmt1, Dnmt2, and Dnmt3 (Dnmt3a, Dnmt3b, Dnmt3L) encoded by different genes have been identified in mammals [Okano et al., 1999; Bestor, 2000; Bourc'his et al., 2001; Hata et al., 2002]. Among these Dnmt2 and Dnmt3L do not demonstrate any enzymatic activity. Dnmt1o, an oocyte-specific isoform of Dnmt1, is essential for the maintenance of some maternally imprinted genes [Howell et al., 2001] whereas Dnmt3L that lacks the catalytic domain co-operates with Dnmt3a and 3b to establish maternal imprinting [Bourc'his et al., 2001; Hata et al., 2002]. Although Dnmt1 is designated maintenance methyltransferase (MTase), and Dnmt3a and 3b are classified as de novo methyltransferases, these enzymes have been shown to exhibit overlapping

Abbreviations used: Dnmt3a/3b/1/2/o/l, DNA methyltransferase3a/3b/1/2/o/l; Dnmt, DNA methyltransferase; HMTase, histone methyltransferase; MTase, methyltransferase; Hdac, histone deacetylase; Rb, retinoblastoma gene product; SAM, S-adenosyl methionine.

Jharna Datta and Kalpana Ghoshal has contributed equally.

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functions [Okano et al., 1998; Rhee et al., 2000; Liang et al., 2002]. In spite of a 5–30 fold preference of Dnmt1 for hemimethylated DNA it exhibits greater de novo DNA methyltransferase (Dnmt) activity in vitro and is present at higher levels than Dnmt3a or 3b in any tissue or somatic cells [Bestor, 2000]. On the other hand, both Dnmt3a and 3b have approximately similar preference for hemimethylated and unmethylated DNA in vitro, are expressed at very low levels in somatic cells and tightly regulated during development [Okano et al., 1998, 1999].

Recent studies have demonstrated that the global hypomethylation and selective hypermethylation at CpG islands of tumor suppressor genes are characteristics of different human malignancies [Feinberg et al., 2002; Jones and Baylin, 2002; Plass, 2002]. The aberrant methylation patterns in most of the cancers is correlated with upregulation of Dnmts [Eads et al., 1999; Robertson et al., 1999; Rountree et al., 2001; Majumder et al., 2002]. The normal activity of the methyltransferases might, however, be dependent upon their cooperation among themselves as well as with other proteins [Robertson et al., 2000; Rountree et al., 2000; Fuks et al., 2001; Kim et al., 2002]. In the past few years our laboratory has been exploring alteration of the methylation machinery in normal and tumor cells [Majumder et al., 2002] and the molecular mechanism of methylation-mediated silencing of genes, particularly metallothionein genes, in cancer cells [Majumder et al., 1999; Ghoshal et al., 2002a,b] and solid tumors [Ghoshal et al., 2000] by promoter methylation.

To elucidate the role of de novo methyltransferases Dnmt3a and 3b in vivo as transcriptional repressors and physical association and/or interaction of these proteins among themselves as well as with other proteins, we fractionated mouse lymphosarcoma S-100 extract through different column matrices and a final glycerol density gradient centrifugation. Our data demonstrate that Dnmt3a can be separated from Dnmt1and Dnmt3b whereas Dnmt3b co-purifies with Dnmt1. We also show that Dnmt3a co-purifies with Hdac1 and a histone H3 methyltransferase (HMTase).

MATERIALS AND METHODS

Generation of Antibodies

Antibodies against recombinant Dnmt3a and 3b were raised in rabbits and purified through antigen-affinity column as described [Majumder et al., 2002]. Antibodies against Hdac1 and Suv39h1 were purchased from Upstate, MA, USA whereas Hdac2 and Hdac3 were from Santa Cruz, CA, USA and Cell Signaling, MA, USA respectively.

Cell Culture, Preparation of S-100 Extract and Assay of De Novo Dnmt Activity

S-100 extract was prepared from mouse lymphosarcoma cells following published protocol [Mahajan and Thompson, 1990]. The assay of de novo Dnmt was as described [Majumder et al., 2002].

Fractionation of Dnmt3a

Dnmt3a was purified following the scheme described in Figure 1.

Q-Sepharose Fast Flow Column Chromatography

P1798 S-100 extract was dialyzed against Buffer-B (20 mM Hepes, [pH 8.0], 25 mM KCl, 0.1 mM EDTA, 2 mM β-mercaptoethanol, 5 mM $MgCl_2$, 0.5 mM PMSF, and 20% glycerol). The dialyzed protein (700 mg, 35 ml) was then loaded onto a Q-Sepharose Fast Flow (Amersham Pharmacia, NJ, USA) column equilibrated with the same buffer. The bound proteins were eluted with 20 bed volume linear gradient of 25 mM to 1.0 M KCl in Buffer B. Dnmt activity was assayed using 15 μl of alternate fractions from the column with unmethylated double stranded complimentary oligonucleotide containing 11 methylatable CpGs as substrate [Majumder et al., 2002]. Western blot analyses were carried out with 200 μl of alternate fractions with antibodies specific for Dnmt3a, Dnmt3b, and Dnmt1, respectively.

Mono-S Column Chromatography

The fractions (# 33–39) containing Dnmt3a (based on Western blot analysis) of Q-Sepharose column were pooled and dialyzed against Buffer B containing 100 mM KCl and the protein was loaded onto a Mono-S FPLC column. The bound protein was eluted with 50 bed volume linear gradient (0.1–1.0 M KCl) and 1ml fractions were collected. The Dnmt activity was measured with 15 μl of each fraction and Western blot analyses were also carried out with 60 μl of each fraction.

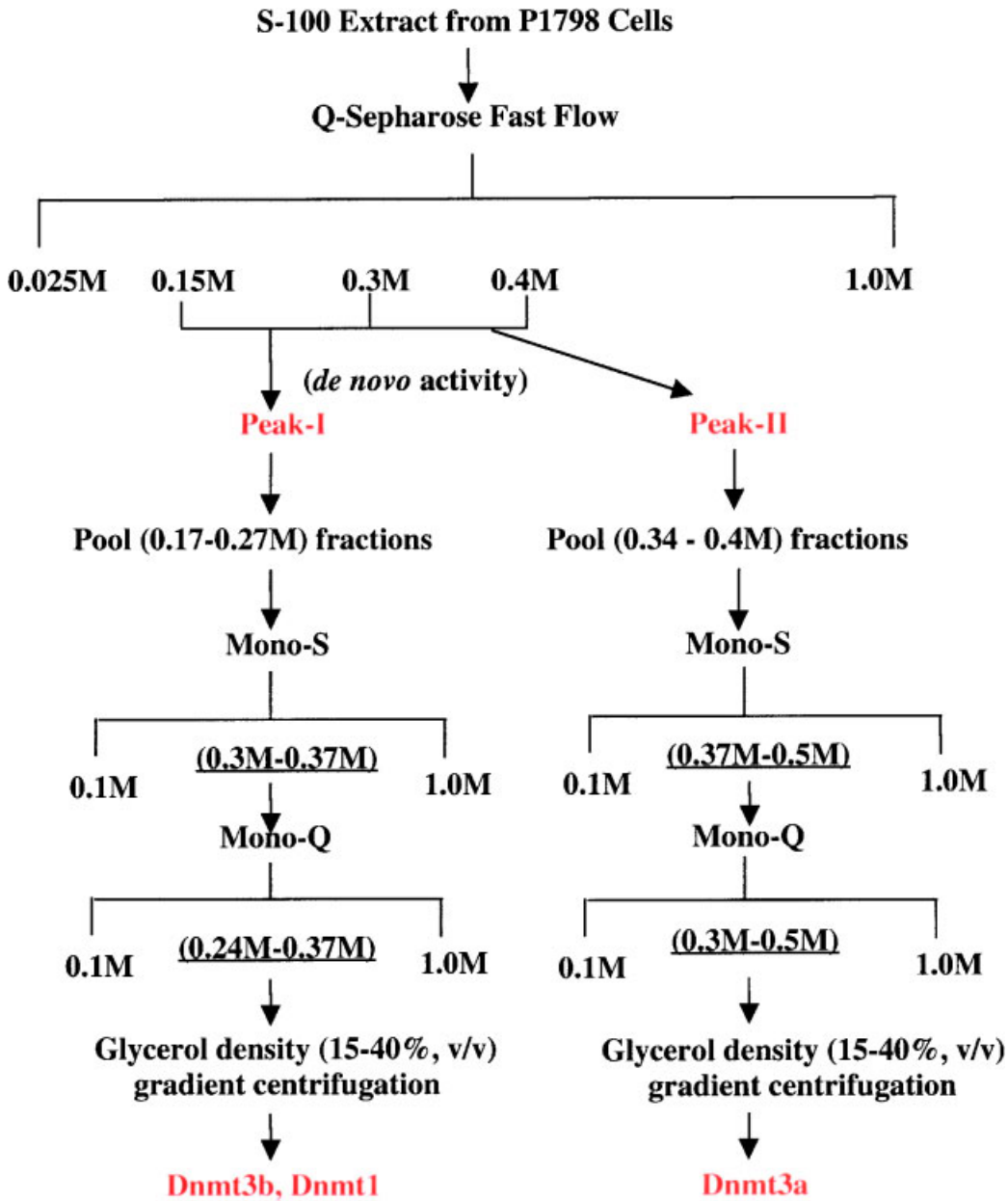

Fig. 1. Schematic representation of the fractionation of Dnmt3a and Dnmt3b.

Mono-Q Column Chromatography

The fractions (#15–27) containing Dnmt3a (based on Western blot analysis) of Mono-S column were pooled, dialyzed against Buffer B containing 100 mM KCl and subjected to chromatography on a Mono-Q FPLC column (1 ml, HR 5/5; Pharmacia). The bound protein was then eluted with 50 bed volume of a linear gradient (0.1–1.0 M KCl) and 1 ml fractions were collected. Dnmt activity was measured with 15 μl of each fraction and Western blot analyses were carried out with 60 μl of each fraction.

Glycerol Density Gradient Centrifugation

Highly purified fractions (500 μl) from Mono-Q column containing Dnmt3a was fractionated by centrifugation (SW 40 Ti) in a glycerol gradient (15–40%) for 60 h. Fractions (500 μl) were collected from the bottom and subjected to Dnmt assay and immunoblot analysis.

Purification of Dnmt3b

Purification of Dnmt3b was carried out following the protocol described in Figure 1. Fractionation of P1798 S-100 extract through Q-Sepharose Fast Flow column was performed as described for Dnmt3a. Mono-S (Q-Sepharose pool, #16–26), Mono-Q (Mono-S pool, #11–15) column chromatography and subsequent glycerol density gradient centrifugation were performed as described for Dnmt3a.

Silver Staining of Proteins

Proteins from each fraction of glycerol density gradient (200 μl) were resolved on 7.5% SDS–PAGE and silver staining was performed using BioRad's Silver Staining Kit.

Assay of Histone Methyltransferase (HMTase) Activity

The HMTase activity was measured either with chicken histones or human histone H3 N-terminal 20 amino acid peptide as described [Rea et al., 2000].

RESULTS AND DISCUSSION

Fractionation of Dnmt3a From Dnmt3b and Dnmt1

Dnmt3a and 3b were cloned by searching database with bacteriophage type II cytosine methyltransferase [Okano et al., 1998] and subsequently the recombinant proteins were expressed in bacteria or insect cells [Gowher and Jeltsch, 2001; Kim et al., 2002]. The endogenous de novo MTase complexes have not been purified from any mammalian cells or tissues probably due to their relatively low abundance/activity compared to Dnmt1 in somatic cells [Okano et al., 1998] and non-availability of specific antibodies until recently. In the present study we purified Dnmt3a and 3b complexes from mouse lymphosarcoma (P1798) cells that can be grown in large number in suspension culture and where the abundance of these proteins is relatively high. To monitor the biochemical fractionation of Dnmt3a in P1798 cell extract the de novo Dnmt assay and Western blot analysis were carried out throughout the purification procedure. We raised specific antibodies against the N-terminal domain of the recombinant mouse Dnmt3a and 3b isoforms expressed as His-tag protein in bacteria [Majumder et al., 2002]. To avoid cross reactivity among Dnmts we deleted the C-terminal catalytic domain shared by all isoforms [Okano et al., 1998]. The authenticity of the antibodies was confirmed by immunoblot analysis against the recombinant full-length isoforms cloned into bacculovirus vector and expressed in Sf-9 cells (data not shown). Antibodies against Dnmt1 were prepared as described [Takagi et al., 1995].

Following fractionation through three different columns using a linear salt gradient throughout (schematically shown in Fig. 1) we were able to separate Dnmt3a from the other two isozymes. When S-100 extract from P1798 cells was fractionated on a Q-Sepharose fast flow column the de novo Dnmt activity was separated into two peaks (Fig. 2A). The activity of peak-I was much higher than that of peak-II. Western blot analyses of the column fractions showed that Dnmt1 and Dnmt3b were eluted in the peak-I whereas Dnmt3a predominantly appeared in peak-II. The antibody against Dnmt1 detected only one polypeptide of ~230 kDa whereas the affinity purified Dnmt3b antibody detected three major polypeptides of ~102, 98, and 85 kDa. These three polypeptides identified by Dnmt3b antibodies are likely to be alternatively spliced forms of Dnmt3b that are detected in different cell types [Chen et al., 2002]. For further purification of Dnmt3a, we pooled together the fractions #33–39 that contained maximum amount of this protein well separated from Dnmt1 and Dnmt3b, and loaded onto a Mono-S column. The de novo Dnmt activity was eluted in a single peak from this column (Fig. 2B, upper panel) although the total enzyme activity of the fractions was much less than that from the previous column. Western blot analyses of the fractions showed that the activity correlated well with a 130 kDa polypeptide corresponding to Dnmt3a (Fig. 2B, lower panel). Dnmt 3a was completely separated from Dnmt3b and Dnmt1 whereas it co-purified with Hdac1. RT-PCR analysis demonstrated that Dnmt3a level was at least five fold less than that of Dnmt3b (data not shown) which was also reflected at the protein and the activity levels. The lower activity of the peak in this column might also be due to separation of Dnmt3a from Dnmt1 and Dnmt3b or because of distributive nature of this isoform [Gowher and Jeltsch, 2001]. To purify Dnmt3a further, we fractionated the pooled Mono-S fractions (#15–27) containing maximal amount/activity of the enzyme through Mono-Q column.

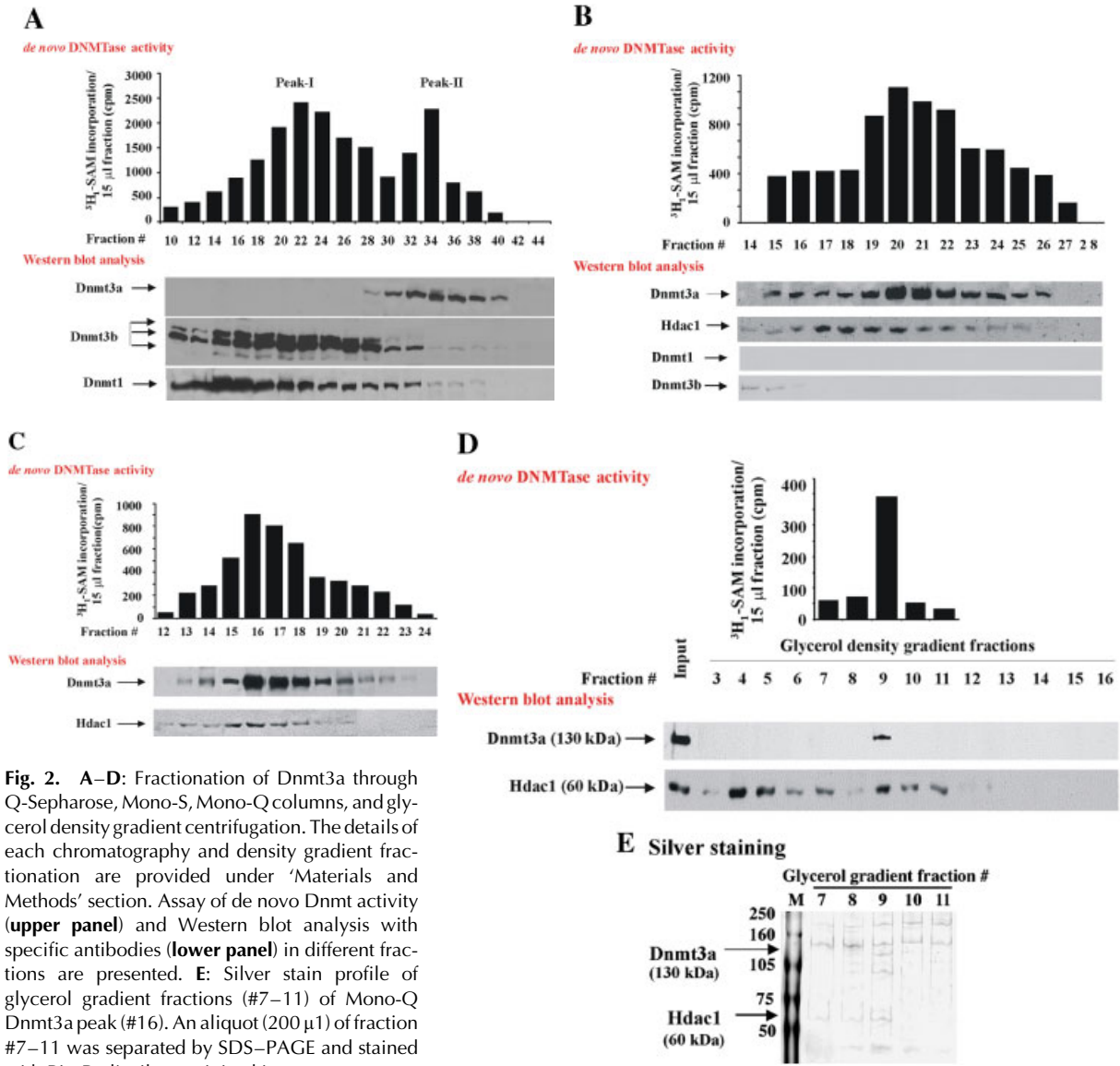

Fig. 2. **A–D**: Fractionation of Dnmt3a through Q-Sepharose, Mono-S, Mono-Q columns, and glycerol density gradient centrifugation. The details of each chromatography and density gradient fractionation are provided under 'Materials and Methods' section. Assay of de novo Dnmt activity (**upper panel**) and Western blot analysis with specific antibodies (**lower panel**) in different fractions are presented. **E**: Silver stain profile of glycerol gradient fractions (#7–11) of Mono-Q Dnmt3a peak (#16). An aliquot (200 µ1) of fraction #7–11 was separated by SDS–PAGE and stained with Bio-Rad's silver staining kit.

The de novo Dnmt activity of the fractions (Fig. 2C, upper panel) correlated with Dnmt3a protein level (Fig. 2C, lower panel). Further, the fractionation profile of Hdac1 was similar to that of Dnmt3a from this column. To separate Dnmt3a complex from other polypeptides we next subjected the peak Mono-Q fraction (#16) to glycerol density gradient centrifugation. De novo Dnmt activity peaked at fraction #9 (#1 being the bottom of the gradient) (Fig. 2D, upper panel) which was the only fraction detected by Dnmt3a antibodies in the Western blot analysis (Fig. 2D, lower panel). The same blot was re-probed with antibodies against Hdac1, Hdac2, and Hdac3 which revealed two peaks of Hdac1, one at fraction #4 and the other at fraction #9, the same fraction where Dnmt3a peaked (Fig. 2D, lower panel). On the other hand, Hdac2 was detected only in fraction #4 and Hdac3 was not detectable in lymphosarcoma cell extract (data not shown). The appearance of Hdac1 in two different fractions in the density gradient indicates that Hdac1 is associated with different complexes in these fractions. Co-sedimentation of Dnmt3a with a population of Hdac1 is in agreement with an earlier report demonstrating direct interaction between Dnmt3a and Hdac1 by GST-pull-down assay and co-immunoprecipitation studies [Fuks et al., 2001]. Next we subjected an aliquot of these fractions to silver staining to identify the molecular size of the polypeptides associated

with Dnmt3a (Fig. 2E). Among these, the ~130 kDa polypeptide represents Dnmt3a, and ~60 kDa corresponds to Hdac1. Other polypeptides have not been identified. Sufficient quantity of other polypeptides should be generated for further identification by mass spectrometry, which is beyond the scope of the present study.

Co-Purification of Dnmt3b With Dnmt1

Next, we fractionated the de novo Dnmt activity corresponding to the first peak of the Q-Sepharose fast flow column (Fig. 2A, upper panel). The higher activity of Peak-I was probably due to the presence of both Dnmt1and Dnmt3b and also due to higher abundance of Dnmt3b compared to Dnmt3a in lymphosarcoma cells (Fig. 2A, lower panel). Immunoblot analysis with affinity purified antibodies detected three different variants of Dnmt3b among which the abundance of the two high molecular mass forms were comparable whereas the expression of the third isoform was quite low. This is consistent with the known alternative splicing of Dnmt3b gene (8). To further purify Dnmt3b and separate it from Dnmt1, the fractions (#16–26) containing the peak activity were pooled, dialyzed, and fractionated on a Mono-S column (see Materials and Methods). The de novo MTase activity peaked at fraction #17 (Fig. 3A, upper panel). The activity of the peak was, however, higher than that of peak-I of Q-Sepharose column indicating significant purification as well as probable dissociation of an inhibitor of de novo Dnmt during fractionation through Mono-S column. Western blot analyses of the fractions with specific antibodies demonstrated that Dnmt1peaked at fraction #17 that correlated with the activity peak whereas Dnmt3b peaked in fraction #15 (Fig. 3A, lower panel). The peak activity correlating with Dnmt1 is expected as in vitro this isoform demonstrates substantial de novo MTase activity [Bestor, 2000]. The robust activity of fractions #16–17 may also arise from the cooperativity of Dnmt3b and Dnmt1. All three isoforms of Dnmt3b were bound to the Mono-S column among which the larger two coeluted whereas the third one eluted earlier (Fraction #10). Further purification of Dnmt3b was accomplished by chromatography of the pooled fractions #11–15 from Mono-S column through a Mono-Q column. De novo Dnmt activity eluted in three fractions, the peak appearing in fraction #9 (Fig. 3B, upper panel) which correlated with the peak of Dnmt3b as analyzed by Western blot (Fig. 3B, lower panel). Interestingly, the peak fraction (#9) also contained a small but detectable level of Dnmt1 indicating close association of these two isoforms in vivo. The peak fractions from Mono-Q column had lower de novo Dnmt activity as compared to those from the Mono-S column which is probably due to removal of the majority of Dnmt1 in the Mono-S column fractions, and lower de novo Dnmt activity in vitro of Dnmt3b compared with Dnmt1 [Bestor, 2000]. Western blot analysis demonstrated that Hdac1 was not detectable in Dnmt3b or Dnmt1 peak fractions from Mono-S column and eluted in later fractions (#11–20) (Fig. 3B). Hdac2 was not detected in any fraction from this column (data not shown). These results indicate that the two major isoforms of Dnmt3b expressed in lymphosarcoma extract are not associated with Hdac1 or 2. Fuks et al. [2001] reported an Hdac activity that was associated with recombinant Dnmt3b overexpressed and immunoprecipitated from cells in culture although the isoform of Hdac has not been explored. Our fractionation studies indicate that Hdac1, 2, and 3 are unlikely to be a component of Dnmt3b complex. To investigate further that Dnmt3b is indeed associated with Dnmt1 the peak fraction was subjected to glycerol density gradient centrifugation and the de novo Dnmt activity and the levels of Dnmt were measured as described for Dnmt3a. The results showed that the de novo Dnmt activity of fractions correlated well with the Dnmt3b and Dnmt1 protein levels (Fig. 3C). The higher activity of Dnmt3b peak in glycerol gradient compared to that of Dnmt3a (Fig. 2D) might be due to its higher abundance, its association with Dnmt1 or processive nature of its catalytic domain [Gowher and Jeltsch, 2002].

Earlier study that focussed on purification of Dnmt1 has shown the existence of a population of Dnmt1 in a complex with eight other polypeptides in HeLa cells, which include retinoblastoma (Rb) tumor suppressor and Hdac1 [Robertson et al., 2000]. Similarly, Dnmt1 has been shown to interact with Hdac2 by yeast two hybrid system [Rountree et al., 2000]. In the present study, none of the three Hdac isoforms tested, Hdac1, 2, and 3 was detected in these fractions by Western blot analyses indicating the lack of association of Dnmt3b/Dnmt1 with these three isoforms of Hdac in lymphosarcoma cells. The major Dnmt1 peak in Mono-S fraction

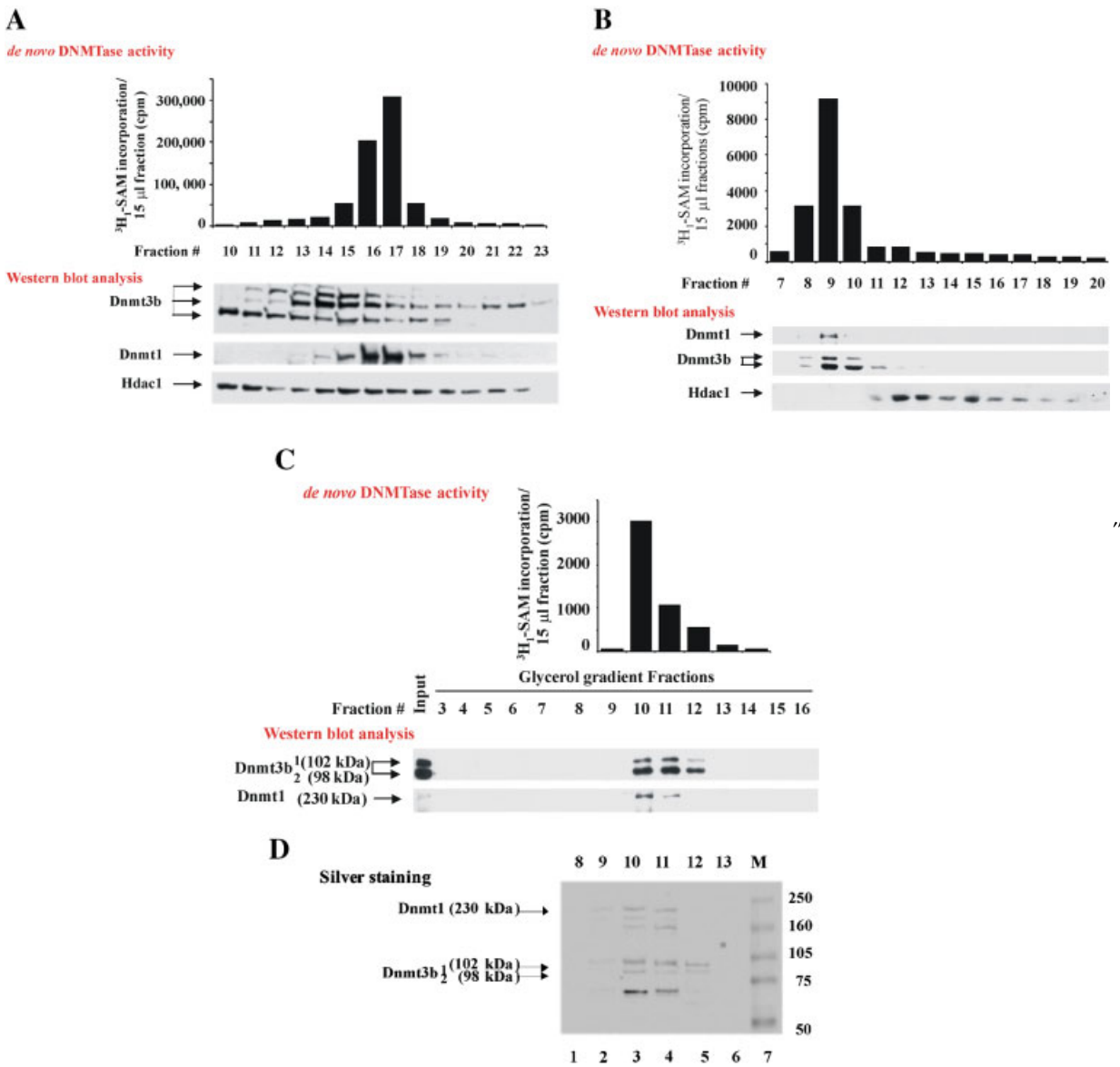

Fig. 3. **A–C**: Fractionation of peak-I (Dnmt3b and Dnmt1) of Q-Sepharose column through Mono-S, Mono-Q, and glycerol density gradient centrifugation The details of each chromatography and density gradient fractionation are provided under 'Materials and Methods' section. De novo Dnmt activity (**upper panel**) and Western blot analysis (**lower panel**) are presented. **D**: Silver stain profile of glycerol gradient fractions (#8–13, 200 µl) of Mono-Q Dnmt 3b peak (#9).

from the lymphosarcoma cells had associated Hdac1 that would probably be retained upon subsequent fractionation. As the aim of the present study was to purify Dnmt3b, the peak Dnmt1 fractions from Mono-S column were avoided for further fractionation. Despite this effort, one population of Dnmt1 co-purified with Dnmt3b even after density gradient fractionation (Fig. 3C). At least the population of Dnmt1 associated with Dnmt3b was devoid of Hdac1 and 2. The ratio of Dnmt1 to Dnmt3b was not significantly reduced after fractionation of Mono-Q peak through glycerol gradient centrifugation, implying that Dnmt1 was not a contaminant associated with Dnmt3b. Five polypeptides other than Dnmt3b ranging in size from ~230 to ~70 kDa were detected in fractions 10 and 11 from glycerol gradient by

silver staining (Fig. 3D). The 230 kDa band however, is Dnmt1, matches with Western blot data (Fig. 3C). The identity of the associated polypeptides has not been established. It should be noted that the only published report to date on the biochemical fractionation of Dnmts was confined to Dnmt1 from HeLa cells [Robertson et al., 2000]. This study focused on the major population of Dnmt1which was probably separated from the minor Dnmt1 form in a complex with Dnmt3b. An important issue here is that Dnmt1 purified from HeLa cells contained as much eight polypeptides in the molecular size ranging from 200 to 54 kDa following the final glycerol density gradient fractionation. The number of polypeptides associated with Dnmt1/Dnmt3b complex from mouse lymphosarcoma cells is comparable to that present in the Dnmt1 preparation from HeLa cells. These results clearly demonstrate close association of Dnmt3b with Dnmt1 in mouse lymphosarcoma cells. These data are consistent with the report that both Dnmt1 and Dnmt3b are essential to maintain the methylation status of genes with CpG island, imprinted genes and of some repeat elements in a human colon cancer cell line implicating functional cooperativity among these enzymes [Rhee et al., 2002].

Association of Dnmt3a With a Histone H-3 (K9) MTase Activity

The observation that deletion of histone H3 lysine-9 (K9) MTase gene (*dim5*) causes inhibition of DNA methylation in *Neurospora crassa* [Tamaru and Selker, 2001] prompted us to check whether Dnmt complexes fractionated from P1798 extract is associated with HMTase activity. Methylation of histone H3 can occur on K4, K9, K27, and K36 that is catalyzed by enzymes with a 130 amino acid signature motif (SET domain) essential for catalysis [Lachner et al., 2001]. To investigate whether a H3 MTase activity cofractionated with Dnmt activity, we measured the HMTase activity in the glycerol gradient fractions containing Dnmt3a or 3b with chicken histones as substrate and $^{3}H_1$-SAM and methyl group donor and $^{3}H_1$-incorporation in histones was measured (see Materials and Methods). The results showed that a HMTase activity cofractionated with Dnmt3a in glycerol gradient fractions of Dnmt3a and the peak HMTase activity coeluted with Dnmt3a (Fig. 4A). In contrast, Dnmt3b fractions from glycerol gradient or Mono-Q column did not exhibit any detectable HMTase activity indicating that Dnmt3b was not associated with any HMTase activity (data not shown). Methylation of histone H3 lysine is catalyzed by different enzymes, some of which are absolutely specific for the site of methylation. For example, Suv39h1, a mammalian homologue of *Drosophila* position-effect variegation modifier [Su (var)3–9] is a K9-specific H3 methyltransferase that can methylate only K9 whereas others can methylate lysines at different sites [Tamaru and Selker, 2001]. To identify the lysine of histone H3 methylated by this HMTase we used glycerol density gradient peak fraction #9 as the enzyme source and 20 amino acid N-terminal histone H3 wild type or mutant peptides as substrate. The activity was abolished when K9-L mutant was used as substrate whereas K4-L mutant inhibited the activity by 35% (Fig. 4B). These results demonstrate that the histone H3 MTase associated with Dnmt3a preferentially methylates lysine 9 of histone H3. At least 70 different SET domain proteins have been identified in mammalian genome [Lachner et al., 2001]. To test whether H3 MTase associated with Dnmt3a is Suv39h1 we performed Western blot analysis of the glycerol gradient fractions as well as in S-100 extract with antibodies specific for this protein but could not detect the specific polypeptide in lymphosarcoma cells (data not shown). The identity of this H3 MTase remains to be determined which is beyond the scope of the present study.

CONCLUDING REMARKS

The two key observations of the present study are (a) Dnmt3b purified through three columns (all using linear salt gradients) and glycerol density gradient, is associated with Dnmt1 which appears to be distinct from the major population of Dnmt1. (b) Dnmt3a is associated with Hdac1 and a HMTase that preferentially methylates K9 of H3. Identification of this HMTase poses a challenge as it appears to be distinct from Suv39h1 and many HMTase in mammalian cells. Similarly, it would be of interest to determine how Dnmt1 associated with Dnmt3b differs from the major population of Dnmt1. The identity of other polypeptides associated with Dnmt3a and 3b purified through glycerol gradient may show how these enzymes are targeted to different regions of

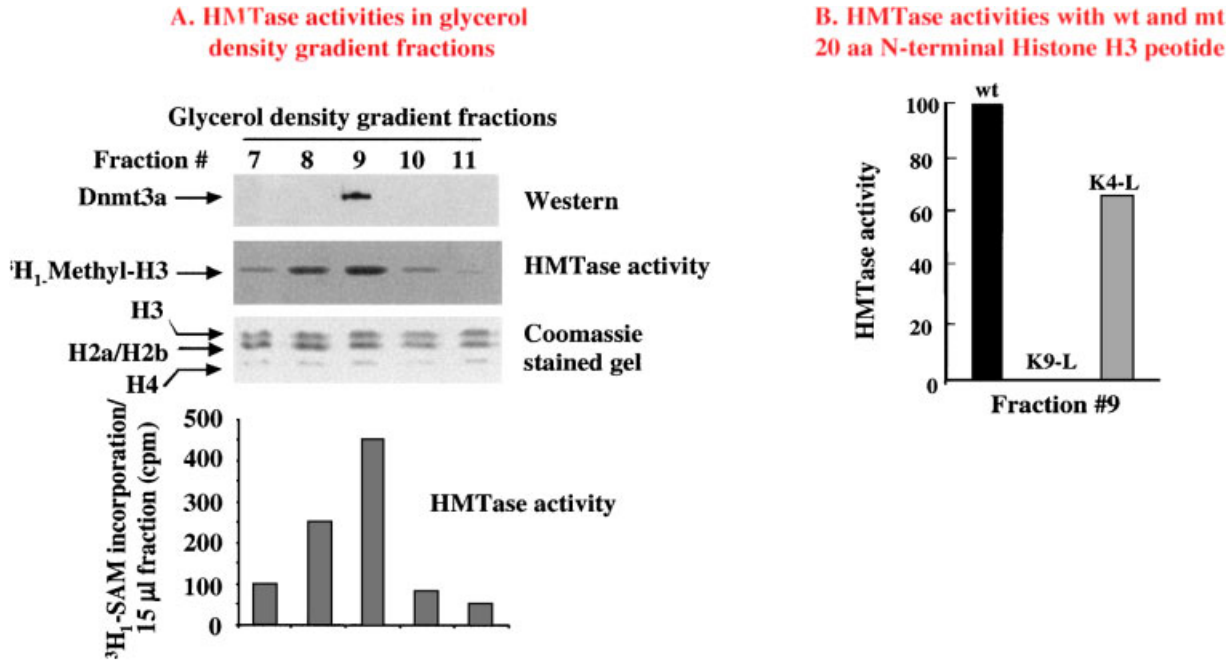

Fig. 4. HMTase activity in glycerol density gradient fractions containing Dnmt3a. **A**: The first four panels represent Western blot analysis of Dnmt3a, fluorography of $^3H_1$-methyl chicken histone H3, Coomassie stain profiles of chicken histones and HMTase activity ($^3H_1$-incorporation as measured by filter binding assay) with chicken histones, respectively. **B**: Histone H3 MTase activity with the wild type and mutant (K9-L or K4-L) histone H3 N-terminal 20 amino acid peptides as substrate in fraction #9 of glycerol gradient fractions.

the chromatin and whether they interact with chromatin remodeling complexes or methyl CpG binding proteins. Investigation along these lines is in progress. Our study has precluded the association of these enzymes with Rb or p53 (data not presented).

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