

Chlamydial SET domain protein functions as a histone methyltransferase

Masayuki Murata,¹ Yoshinao Azuma,¹ Koshiro Miura,^{1,2†}
Mohd. Akhlakur Rahman,¹ Minenosuke Matsutani,¹ Masahiro Aoyama,¹
Harumi Suzuki,¹ Kazuro Sugi² and Mutsunori Shirai¹

Correspondence
Yoshinao Azuma
yazuma@yamaguchi-u.ac.jp

Mutsunori Shirai
mshirai@yamaguchi-u.ac.jp

¹Department of Microbiology and Immunology, Yamaguchi University School of Medicine, 1-1-1 Minami-Kogushi, Ube, Yamaguchi 755-8505, Japan

²Department of Clinical Research, National Sanyo Hospital, 685 Higashi-Kiwa, Ube, Yamaguchi 755-0241, Japan

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SET domain genes have been identified in numbers of bacterial genomes based on similarity to SET domains of eukaryotic histone methyltransferases. Herein, a *Chlamydomphila pneumoniae* SET domain gene was clarified to be coincidentally expressed with *hctA* and *hctB* genes encoding chlamydial histone H1-like proteins, Hc1 and Hc2, respectively. The SET domain protein (cpnSET) is localized in chlamydial cells and interacts with Hc1 and Hc2 through the C-terminal SET domain. As expected from conservation of catalytic sites in cpnSET, it functions as a protein methyltransferase to murine histone H3 and Hc1. However, little is known about protein methylation in the molecular pathogenesis of chlamydial infection. cpnSET may play an important role in chlamydial cell maturation due to modification of chlamydial histone H1-like proteins.

INTRODUCTION

Chlamydomphila pneumoniae is an obligatory intracellular eubacterium that causes acute respiratory diseases and may be involved in chronic inflammatory processes, such as atherosclerosis (Rosenfeld *et al.*, 2000), asthma (Hahn *et al.*, 1991) and Alzheimer's disease (Itzhaki *et al.*, 2004). Persistence of chlamydial infection has been thought to be important for chronic diseases and has been characterized using model animals and activation stimuli such as cytokines and antibiotics (Beatty *et al.*, 1993; Belland *et al.*, 2003; Malinverni *et al.*, 1995; Mehta *et al.*, 1998). However, molecular-level relationships between chronic disease progression and persistent infection are not yet clear.

Chlamydiae exhibit a unique life cycle in which they alternate morphologies between elementary bodies (EBs) and reticulate bodies (RBs). EBs are transcriptionally inactive electron-dense particles that are internalized into host cells by inducing phagocytosis. EB differentiation into RBs occurs with the development of phagosomes

into inclusions. Transcriptionally active RBs multiply by binary fission with nutrients acquired from the host cell. At the end of the developmental cycle, RBs are converted into EBs and released from host cells for the next infection. Besides the developmental cycle, during persistent infection caused by exposure to interferon gamma (IFN- γ) or antibiotics, RBs differentiate into aberrantly large and non-multiplying RBs (Belland *et al.*, 2003). However, little is known about the switching mechanism whereby vegetative RBs convert into infectious EBs or aberrant RBs. Understanding this molecular system should be helpful for the prevention of persistent chlamydial infection.

Two eukaryotic histone H1-like proteins of chlamydiae, Hc1 and Hc2, are present mainly in EBs, where those proteins bind DNA and promote genomic DNA condensation (Barry *et al.*, 1992; Hackstadt *et al.*, 1991; Perara *et al.*, 1992; Tao *et al.*, 1991). Recently a small regulatory RNA gene was identified as a suppressor of the lethal phenotype of *hctA* overexpression in *Escherichia coli* and it was shown to negatively regulate Hc1 synthesis at an early stage of infection (Grieshaber *et al.*, 2006). These histone-like proteins may act as global transcriptional regulators and play a critical role for the transformation of vegetative RBs into infectious EBs. Transcriptional, translational and functional regulations of Hc1 and Hc2 may be important for the morphological switching. Chlamydial genome analyses have revealed the existence of another candidate gene as a regulator of Hc1 and Hc2, termed the *set* gene,

†Present address: Department of Veterinary Biosciences, the Ohio State University, 1900 Coffey Rd, Columbus, OH 43210, USA.

Abbreviations: cpnSET, *Chlamydomphila pneumoniae* SET domain protein; EB, elementary body; RB, reticulate body; FCS, fetal calf serum; GST, glutathione S-transferase; h.p.i., hours post-infection.

A supplementary table and three supplementary figures are available with the online version of this paper.

which encodes a protein containing a domain similar to the eukaryotic SET domain (Stephens *et al.*, 1998). Eukaryotic SET domains were initially identified in the C-terminal ends of *Drosophila* transcriptional regulatory factors (Alvarez-Venegas & Avramova, 2002; Jones & Gelbart, 1993; Kouzarides, 2002; Kuzmichev *et al.*, 2005) and have been shown to be involved in chromatin remodelling due to histone methyltransferase activity to specific residues in amino-terminal histone tails, such as histone H3 K9 and K27 (Marmorstein, 2003.; Xiao *et al.*, 2003).

Herein, we demonstrate that the chlamydial SET domain protein physically interacts with chlamydial histone-like proteins Hc1 and Hc2, and functions as a histone methyltransferase to methylate mouse histone H3 and Hc1. The results suggest involvement of the SET domain protein in chlamydial cell transformation from RBs to EBs.

METHODS

Chemicals, cell line and bacterial strains. Gentamicin, cycloheximide, Hoechst 33258 and Dulbecco's modified Eagle medium were purchased from Sigma-Aldrich. Fetal calf serum (FCS) was from Cansera International. Anti-*C. pneumoniae*-specific monoclonal antibody (RR402) was purchased from Washington Research Foundation. FITC-conjugated goat anti-mouse antibody and Alexa 545-conjugated goat anti-rabbit antibody were obtained from Invitrogen. S-Adenosyl-[methyl-¹⁴C]-L-methionine was purchased from Amersham.

HEp-2 cells (ATCC CCL-23) were used as host cells for infection by *C. pneumoniae* J138, isolated in Japan in 1994 (Shirai *et al.*, 2000). *C. pneumoniae* J138 EBs were purified by sucrose-gradient centrifugation and stored at -80°C in SPG buffer (pH 7.2), which consists of 250 mM sucrose, 10 mM sodium phosphate and 5 mM glutamate. Chlamydial titres were adjusted to 2.0×10^8 inclusion-formation units (i.f.u.) ml^{-1} .

Chlamydial infection. Chlamydial infections were performed by methods described previously (Rahman *et al.*, 2005). Briefly, HEp-2 cells were grown in HEp-2 medium (Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated FCS and 50 μg gentamicin ml^{-1}) at 37°C , 5% CO_2 . Prior to infection, 2.0×10^5 HEp-2 cells were seeded to each well of six-well tissue culture plates and allowed to adhere for 24 h. Infection was performed by addition of *C. pneumoniae* J138 EBs at 0.20 m.o.i., followed by centrifugation at 22°C for 60 min at 700 g. After incubation for 30 min at 36°C , 5% CO_2 , the inocula were replaced with post-infection medium (Dulbecco's modified Eagle medium with 5% heat-inactivated FCS, 50 μg gentamicin ml^{-1} and 1 μg cycloheximide ml^{-1}). The infectants were incubated for up to 72 h at 36°C , 5% CO_2 .

Vector construction. pGEX(2T-P)+cpnSET full-length and pGEX(2T-P)+cpnSET (206–219 aa) were constructed by cloning the *C. pneumoniae* J138 *set* gene fragments into pGEX(2T-P) (Azuma *et al.*, 1995). Three deletion series of pGEX(2T-P)+Hc1 [Hc1-1 (aa 1–50), Hc1-2 (aa 41–78) and Hc1-3 (aa 65–123)] were constructed based on the pGEX(2T-P)+Hc1. For the yeast two-hybrid study, pGBKT7+cpnSET was prepared by cloning *C. pneumoniae* J138 *set* gene into pGBKT7 (Clontech). For construction of pGADT7+Hc1 and pGADT7+Hc2, *C. pneumoniae* J138 *hctA* and *hctB* genes, respectively, were cloned into pGADT7 (Clontech). Eight deletion series of pGBKT7-cpnSET were constructed by PCR using pGBKT7-cpnSET. pGEX4T-3-mG9a and pGEX4T-3-H3, encoding GST fusion G9a (621–1000 aa) and GST fusion histone H3 (1–50 aa), respectively, were kind gifts of Professor Yoichi Shinkai

(Kyoto University, Kyoto, Japan) (Tachibana *et al.*, 2001). All primers used in this work are shown in Supplementary Table S1, available with the online version of this paper.

Preparation of recombinant proteins and anti-cpnSET anti-serum. GST fusion proteins were produced in *E. coli* JM109 cells and purified using glutathione-agarose affinity purification in lysis buffer [20 mM Tris/HCl (pH 8.0), 5 mM EDTA, 0.5% Triton X-100, 0.2 mM PMSF and a protease inhibitor mixture] (Azuma *et al.*, 1993). One milligram of GST fusion cpnSET protein was cleaved with 0.02 U thrombin (Novagen) in thrombin reaction solution [20 mM Tris/HCl (pH 8.4), 150 mM NaCl, 2.5 mM CaCl_2] for 2 h at 20°C , and the thrombin was removed by incubation with 50 μl *p*-aminobenzamidene-agarose beads (Amersham) for 1 h at 4°C . Anti-cpnSET rabbit polyclonal sera were prepared by immunization of rabbits five times every other week with 0.1 μg of the purified GST fusion cpnSET (aa 206–219) protein, following the method described previously (Miura *et al.*, 2001).

Histochemical analysis. After fixation with 100% methanol for 60 min, the infectants were incubated with anti-*C. pneumoniae*-specific monoclonal antibody (RR402) and anti-cpnSET rabbit serum as described above for 60 min at 25°C . After washing, cells were stained with FITC-conjugated goat anti-mouse antibody and Alexa 545-conjugated goat anti-rabbit antibody. Nucleic acids were stained with 2 μg Hoechst 33258 ml^{-1} for 10 min. Microscopic observation was performed with an LSM510 laser scanning confocal microscope (Zeiss).

Quantitative RT-PCR. For quantitative RT-PCR by a LightCycler (Roche), QuantiTect SYBR Green RT-PCR (Qiagen) was used with a total RNA fraction extracted from *C. pneumoniae*-infected cells. Reactions were performed based on the manufacturer's instructions. All primers are shown as a supplementary table within the online version of this paper at Table S1 for primers.

Protein interaction analyses. Yeast two-hybrid analysis was performed using MatchMaker GAL4 Two-Hybrid System 3 kits (Clontech) according to the manufacturer's instructions. Transformants were assayed by growth on plates without leucine, tryptophan and histidine, and without leucine and tryptophan as a control.

Structure modelling of cpnSET was carried out using the virus SET structure as a template (Eswar *et al.*, 2003; Manzur *et al.*, 2003) and peptide docking analysis onto cpnSET was performed using AutoDock (Morris *et al.*, 1996).

In vitro histone methyltransferase assay. Procedures for *in vitro* measurement of histone methyltransferase activity were adapted from the protocol reported previously (Tachibana *et al.*, 2001). Briefly, the assay was carried out with 0.5 μg mG9a or cpnSET protein and 0.5 μg GST fusion mouse histone H3 or chlamydial Hc1 as a substrate in 50 μl reaction buffer (50 mM Tris/HCl pH 8.5, 20 mM KCl, 10 mM MgCl_2 , 10 mM β -mercaptoethanol, 250 mM sucrose and 4.6 kBq S-adenosyl-[methyl-¹⁴C]-L-methionine as methyl donor). After incubation for 60 min at 37°C , reactions were stopped by addition of 15 μl SDS buffer [6% SDS, 150 mM Tris/HCl (pH 6.8), 300 mM DTT, 0.1% BPB and 30% (v/v) glycerol] and boiling at 100°C for 10 min. Methyl-¹⁴C was detected using a BAS-2000 scanner (FujiFilm) after protein separation by 12% acrylamide SDS-PAGE.

RESULTS

Chlamydial SET domain protein

Chlamydial genome analysis has revealed the existence of a number of genes similar to eukaryotic genes involved in

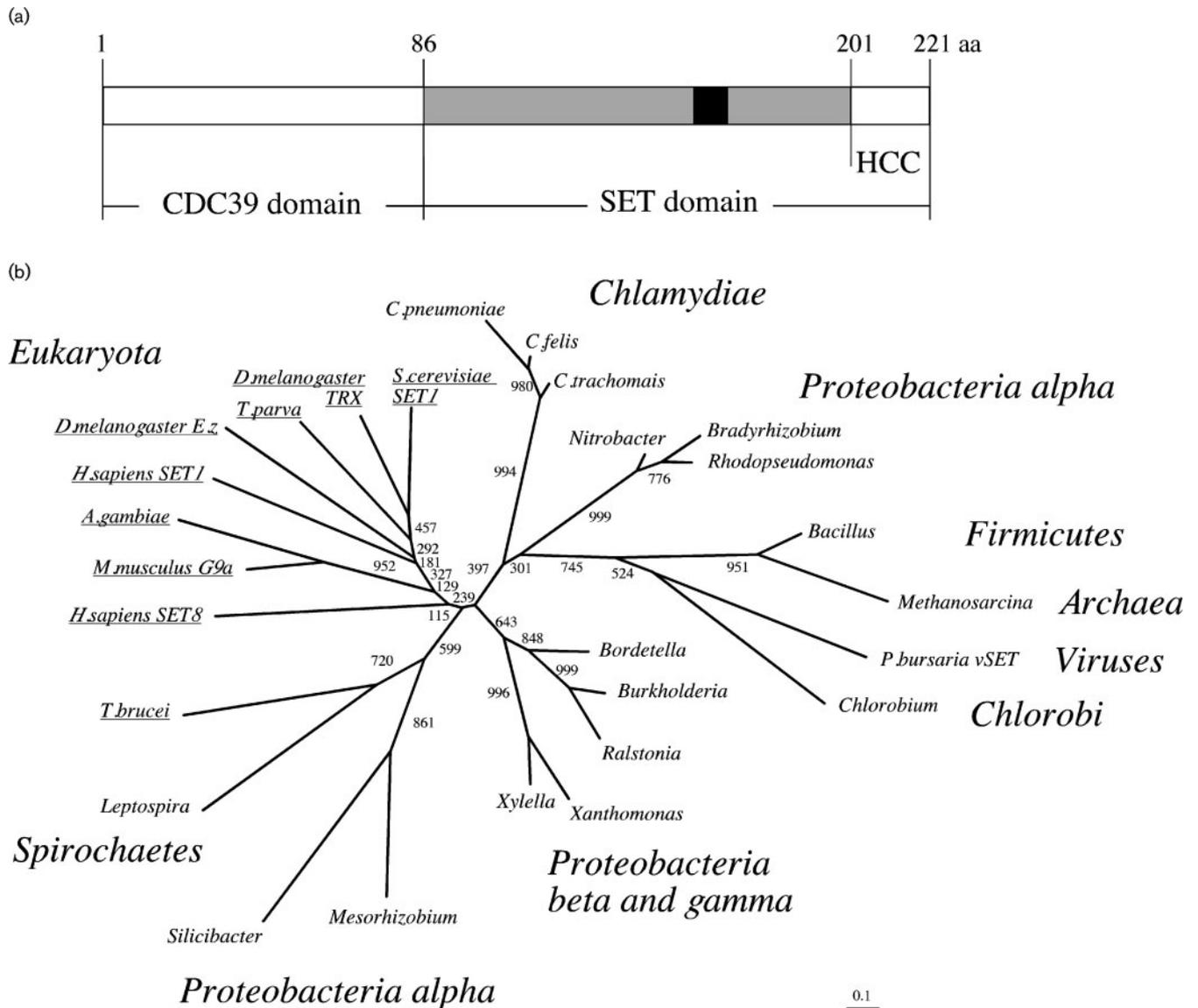


Fig. 1. *C. pneumoniae* SET domain protein (cpnSET). (a) Domains in cpnSET domain protein. The signature motif, 'RFINHCXPN' (Dillon *et al.*, 2005) is indicated with a black box. The HCC in the C-terminus shows a highly charged and conserved (HCC) domain. (b) Phylogenetic analysis of SET-domain proteins based on sequence alignment. The alignment includes the known members of the SET-domain protein family. *C. pneumoniae*, *Chlamydomonas pneumoniae* J138 (NP300935); *C. felis*, *Chlamydomonas felis* Fe/C-56 (CF0125); *C. trachomatis*, *Chlamydia trachomatis* D/UW-3/CX (NP220256); *Bradyrhizobium*, *Bradyrhizobium japonicum* USDA110 (BAC51052); *Rhodopseudomonas*, *Rhodopseudomonas palustris* BisA53 (ZP00807683); *Nitrobacter*, *Nitrobacter winogradskyi* Nb-255 (ABA05415); *Bacillus*, *Bacillus anthracis* Ames (ZP00390019); *Burkholderia*, *Burkholderia cenocepacia* (ZP00459508); *Ralstonia*, *Ralstonia eutropha* JMP134 (AAZ62749); *Bordetella*, *Bordetella pertussis* 12822 (CAE44800); *Xanthomonas*, *Xanthomonas oryzae* MAFF (YP200046); *Xylella*, *Xylella fastidiosa* (AAF84287); *Mesorhizobium*, *Mesorhizobium loti* MAFF303099 (BAB50081); *Silicibacter*, *Silicibacter pomeroyi* DSS-3 (AAV95586); *Chlorobium*, *Chlorobium tepidum* TLS (AAM72187); *Leptospira*, *Leptospira interrogans* serovar Copenhageni str. (AAS71524); *Methanosarcina*, *Methanosarcina mazei* Go1 (AAM32541); *P. bursaria* vSET, *Paramecium bursaria chlorella virus-1* (NP048968); *H. sapiens* SET1, *Homo sapiens* (NP071900); *H. sapiens* SET8, *Homo sapiens* (NP065115); *M. musculus* G9a, *Mus musculus* (NP665829); *D. melanogaster* TRX, *Drosophila melanogaster* (NP599109); *D. melanogaster* E.z., *Drosophila melanogaster* (AAF50149); *S. cerevisiae* SET1, *Saccharomyces cerevisiae* S288C (P38827); *T. parva*, *Theileria parva* Muguga (AN32159); *T. brucei*, *Trypanosoma brucei* TREU927/4 (EAN77879); *A. gambiae*, *Anopheles gambiae* PEST (EAA07914).

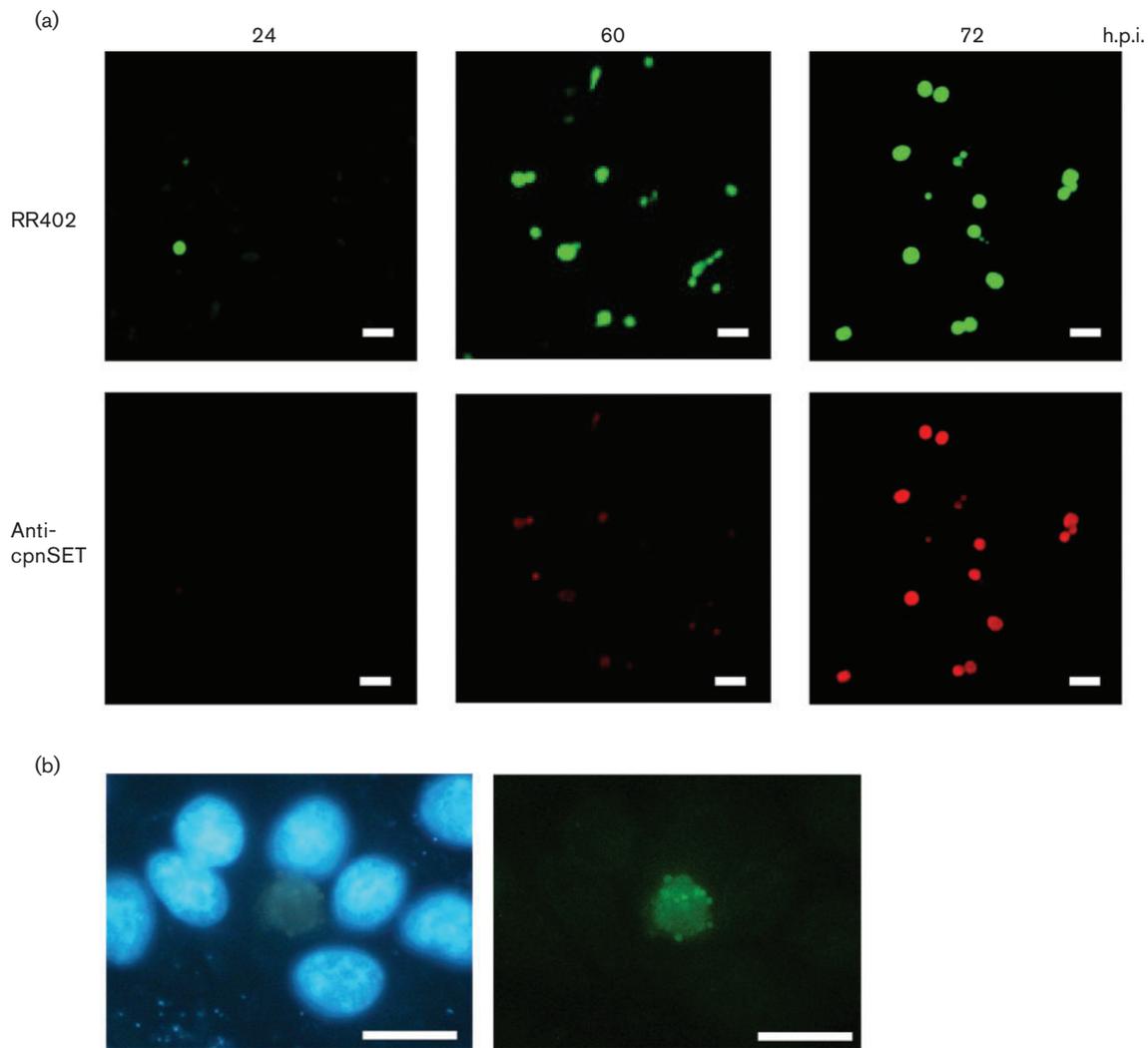


Fig. 2. Expression and localization of cpnSET within *C. pneumoniae* J138-infected HEp-2 cells. (a) Infected cells were fixed at 24, 60 and 72 h.p.i. and stained with the indicated antibodies. The anti-cpnSET is *C. pneumoniae* SET-specific rabbit polyclonal antibody prepared in this work and RR402 is *C. pneumoniae*-specific murine monoclonal antibody used as a control. Magnification, $\times 400$; bars, 10 μm . (b) The left and right panels are Hoechst 33258 and anti-cpnSET rabbit serum staining, respectively. Magnification, $\times 1000$; bars, 5 μm .

chromatin maintenance, such as histone, SET and SWI/SNF (Azuma *et al.*, 2006; Carlson *et al.*, 2005; Kalman *et al.*, 1999; Read *et al.*, 2000, 2003; Shirai *et al.*, 2000; Stephens *et al.*, 1998; Thomson *et al.*, 2005). CPj0878 is assigned as a gene coding a SET domain protein in *C. pneumoniae* J138 and well conserved (overall more than 60% identities) in the family *Chlamydiaceae*. The C-terminal SET domain shows approximately 30% identities to many eukaryotic SET domains. The eukaryotic SET domains are well characterized as catalytic domains of histone methyltransferases involved in chromatin remodelling, especially transcriptional regulation and establishment of heterochromatin (Marmorstein, 2003; Xiao *et al.*, 2003). The chlamydial SET domain protein consists of two distinct domains, CDC39 and SET (Fig. 1a). The N-terminal one-third is partially similar to a CDC39/NotI component in a yeast general

transcription-negative regulator CCR4–Not complex involved in controlling mRNA initiation (Liu *et al.*, 1998). This CDC39 region is well conserved only in chlamydial SET domain proteins but not in other organism SET proteins. The C-terminal two-thirds region preserves SET signature motifs, such as S-adenosyl-L-methionine-binding sites and a catalytic site for the histone methyltransferase activity (Zhang *et al.*, 2003).

The chlamydial SET domain gene (*set*) was thought to be established as a result of horizontal gene transfer from a eukaryotic organism to a chlamydial ancestor (Stephens *et al.*, 1998). But recent progress in genome analysis has revealed the existence of numbers of genes encoding SET-like domains in non-eukaryotic organisms, e.g. *Paramecium bursaria chlorella virus-1* (Manzur *et al.*, 2003), archaeal

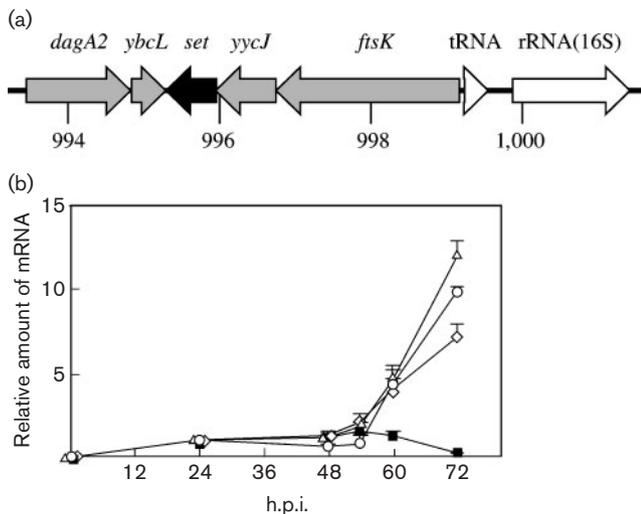


Fig. 3. *C. pneumoniae set* gene. (a) Genome map showing the *set* gene locus. (b) Quantitative RT-PCR of *set*, *hctA*, *hctB* and *ompA* genes. Total RNA was prepared from HEp-2 cells (ATCC CCL-23) infected with *C. pneumoniae* J138 (Rahman *et al.*, 2005) at 0, 12, 24, 48, 54, 60 and 72 h.p.i. Infection was carried out as described previously (Rahman *et al.*, 2005). Expression of *set* (○), *hctA* (△), *hctB* (◇) and *ompA* (■) genes were normalized with 16S rRNA. Relative expression was calculated as the ratio to the value of the expression of each gene at 24 h.

Methanosarcina mezei (Manzur & Zhou, 2005) and a variety of eubacterial phyla such as *Firmicutes*, *Proteobacteria*, *Chlorobi*, *Spirochaetes* and *Chlamydiae*. Phylogenetic analysis of eubacterial and other SETs illustrates that eubacterial SETs diverge into a few groups similar to the general taxological phyla (Fig. 1b). It suggests that chlamydial *set* genes are not transferred horizontally from eukaryotic organisms, but rather likely diverged from a bacterial origin. The sequence alignment of SETs is shown as Supplementary Fig. S1 with the online version of this paper.

Gene expression and protein localization of cpnSET

Since the existence of the *C. pneumoniae* SET domain protein (cpnSET) was proposed based on prediction by genomic analysis, prior to functional analyses, its gene expression and protein localization were investigated by quantitative RT-PCR and immunohistochemical observation. Anti-cpnSET rabbit polyclonal serum prepared in this work was used to detect cpnSET protein in HEp-2 cells infected with *C. pneumoniae* J138. Simultaneously, anti-*C. pneumoniae*-specific monoclonal antibody RR402 and Hoechst 33258 were used for counter-staining. While chlamydial inclusions visualized by RR402 were detectable at any stages, cpnSET was detected only at 60 and 72 h post-infection (h.p.i.) (Fig. 2a) and in chlamydial cells (Fig. 2b). It is a similar expression pattern of Hc1 and Hc2 proteins

encoded by *hctA* and *hctB* genes, respectively (Hackstadt *et al.*, 1991).

In chlamydial genomes, *fisK*, *yycJ* and *set* genes are closely located in this order and seem to constitute an operon (Fig. 3a). In contrast to SET protein accumulation (Fig. 2a), chlamydial *fisK* has been shown to express constantly from middle to late infection stages (Byrne *et al.*, 2001) and our data from microarray analyses for chlamydial expression showed results consistent with this (data not shown). To clarify the expression of *set*, quantitative RT-PCR was performed. Relative expression of *set*, *ompA*, *hctA* and *hctB* normalized to 16S rRNA amount are shown in Fig. 3(b). Constant expression of *ompA* and late-stage expression of *hctA* and *hctB* were consistent with previous reports (Fahr *et al.*, 1995; Slepkin *et al.*, 2003). The accumulation of *set* mRNA was increased simultaneously with *hctA* and *hctB* after 54 h.p.i., suggesting that *set* may be transcriptionally independent of *fisK* and regulated in the same manner as *hctA* and/or *hctB*.

CpnSET interaction with histone-like protein

Physical interaction of cpnSET with Hc1 and Hc2 was tested by the yeast two-hybrid system. It was clarified that cpnSET can interact with Hc1 and Hc2. Based on the experiments using deletion series of cpnSET, the binding to the Hc1 and Hc2 is through aa 137–200 and 137–160, respectively, of cpnSET, both of which regions contain substrate-binding amino acids required for histone methyltransferase activity (Fig. 4a). SET protein localization, expression stage and interaction with Hc1 and Hc2 strongly suggest that cpnSET catalyses protein methylation to Hc1 and Hc2 proteins. The original data for the yeast two-hybrid system are shown as Supplementary Fig. S2 with the online version of this paper.

To know whether cpnSET functions as a methyltransferase, an *in vitro* methyltransferase assay was carried out using recombinant murine histone H3 as a substrate under the conditions optimized for mouse G9a, which is a major mammalian histone methyltransferase containing a SET domain responsible for methylation of K9 in histone H3 at euchromatin (Tachibana *et al.*, 2001). A 0.5 µg sample of cpnSET, GST and G9a proteins was separately incubated with 0.5 µg recombinant mouse histone H3. It was shown that cpnSET can methylate histone H3 and the methylation activity of cpnSET is 14 % of G9a under these conditions (Fig. 4b).

As a substrate candidate of chlamydial proteins for cpnSET activity, Hc1 protein was subjected to this assay with Hc1-1, Hc1-2 and Hc1-3 (aa 1–50, 41–78 and 65–123, respectively). The full lengths of Hc1 and Hc2 are difficult to keep soluble in the processes of protein purification and methyltransferase reaction. As a result, only the Hc1-1 was capable of being methylated by cpnSET (Fig. 4c). No apparently conserved sequences were found between H3 (aa 1–50) and Hc1-1 (Fig. 4c). However, the combination of two informatics analyses, structure modelling of cpnSET using the virus SET

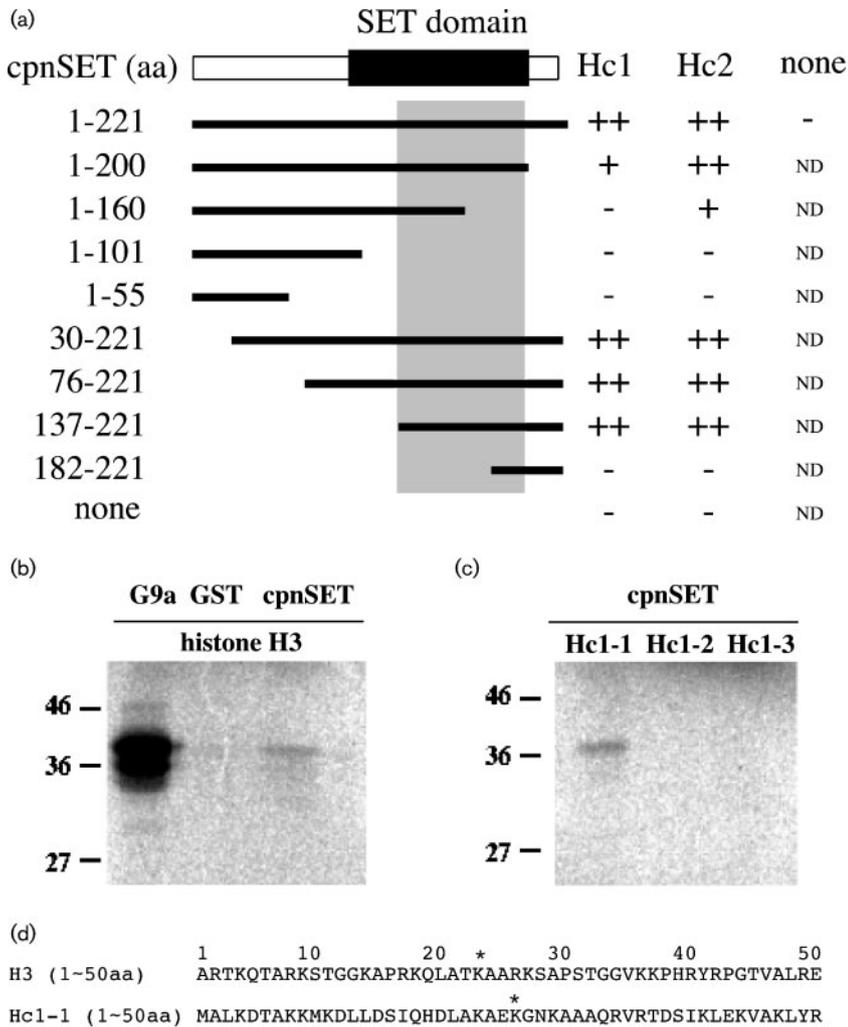


Fig. 4. Interaction of cpnSET and chlamydial histone-like proteins. (a) Two-hybrid assay between cpnSET or its deletion series, and Hc1 and Hc2. The filled box (aa 102–181) and grey region show the SET domain and interaction domain of cpnSET with Hc1 and Hc2, respectively. ++, + and –, good, weak, and no growth, respectively, on culture medium without histidine. None, vector only; ND, not determined. (b) Histone H3 methylation. GST-G9a (indicated as G9a), GST-cpnSET (cpnSET) and GST protein (GST) were subjected to methylation of GST-H3 protein (histone H3). (c) Chlamydial Hc1 methylation. Hc1 deletion proteins Hc1-1, Hc1-2 and Hc1-3 were used as substrates for *in vitro* methylation by chlamydial GST-cpnSET (cpnSET). (d) Alignments of histone H3 and chlamydial histone-like protein, Hc1-1. Histone H3 (CAH73371) and Hc1-1 (NP_300943) are shown 50 aa from the N terminus. The predicted methylated residues are indicated with asterisks.

structure and peptide docking analysis, indicates that K27 of H3, one of the specific methylation sites (Tachibana *et al.*, 2001), and K29 of Hc1 were the best-fitting lysine residues to the catalytic space of cpnSET on the basis of the lowest docking energy. The modelled cpnSET structure and docking profile are shown in Supplementary Fig. S3 with the online version of this paper.

DISCUSSION

Numbers of genes encoding SET domain proteins have been identified in genomes of non-eukaryotic organisms, e.g. *Paramecium bursaria chlorella* virus-1, archaeal *Methanosarcina mezei* and bacterial *Bacillus anthracis*, *Bacillus cereus*, *Xylella fastidiosa*, *Leptospira interrogans*, *Bradyrhizobium japonicum*, *Chlorobium tepidum* and species of the family *Chlamydiaceae*. It has been reported that the viral SET domain protein methylates histone H3 Lys27, and the archaeal SET domain protein methylates lysine residues of eukaryotic histone H4 and archaeal DNA-binding protein MC1- α (Manzur *et al.*, 2003; Manzur & Zhou, 2005). Herein, cpnSET was shown to function as a protein methyltransferase to methylate both murine histone H3

and chlamydial Hc1 *in vitro*, suggesting that cpnSET may play an important role in modification of Hc1 proteins for the morphological change from RBs to EBs. Since localization of cpnSET was shown mainly in chlamydial cells, cpnSET may methylate Hc1 *in vivo*, but it is still possible that chlamydial SET proteins are exported into host cells or that host histones are transported into chlamydial cells, and then cpnSET and host histones functionally interact with each other. Identification of physiological substrates for methylation by cpnSET and, if Hc1 is one of the physiological substrates for methylation *in vivo*, elucidation of the significance of Hc1 protein modification remains for further investigation. Here one of the eubacterial SET domain proteins was revealed as a protein methyltransferase, and this finding suggests that other eubacterial SET domain proteins may function as methyltransferases as well.

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