Phosphorylation Drives an Apoptotic Protein to Activate Antiapoptotic Genes

PARADIGM OF INFLUENZA A MATRIX 1 PROTEIN FUNCTION

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Background: Influenza A matrix 1 protein (M1) associates with nuclear domain 10 (ND10) early in infection.

Results: Phosphorylated M1 upon nuclear translocation induces survival genes by inhibiting death domain-associated protein 6 (Daxx) of ND10.

Conclusion: M1 in early infection exhibits phosphorylation-dependent antiapoptotic function.

Significance: This study uncovers the antiapoptotic role of M1 and identifies a possible therapeutic target to limit virus infection.

During infection, viral proteins target cellular pathways that regulate cellular innate immune responses and cell death. We demonstrate that influenza A virus matrix 1 protein (M1), an established proapoptotic protein, activates nuclear factor-κB member RelB-mediated survival genes (cIAP1, cIAP2, and cFLIP), a function that is linked with its nuclear translocation during early infection. Death domain-associated protein 6 (Daxx) is a transcription co-repressor of the RelB-responsive gene promoters. During influenza virus infection M1 binds to and stabilizes Daxx protein by preventing its ubiquitination and proteasomal degradation. Binding of M1 with Daxx through its Daxx binding motif prevents binding of RelB and Daxx, resulting in up-regulation of survival genes. This interaction also prevents promoter recruitment of DNA methyltransferases (Dnmt1 and Dnmt3a) and lowers CpG methylation of the survival gene promoters, leading to the activation of these genes. Thus, M1 prevents repressional function of Daxx during infection, thereby exerting a survival role. In addition to its nuclear localization signal, translocation of M1 to the nucleus depends on cellular kinase-mediated phosphorylation as the protein kinase C inhibitor calphostin C effectively down-regulates virus replication. The study reconciles the ambiguity of dual antagonistic function of viral protein and potentiates a possible target to limit virus infection.

Virus infection and replication are often associated with apoptosis, and this effect is responsible for much of the pathology associated with infectious disease. Viruses have developed diverse mechanisms to counteract or delay the apoptotic process initiated by the cell’s innate immune responses, thereby prolonging the life of the infected cell such that virus replication, spread, and persistence is maximized (1, 2). These viral proteins target the cellular pathways responsible for regulating apoptosis. In mammalian cells, apoptotic pathways involve caspases, a diverse family of cysteinyl aspartate proteases, through which apoptosis initiation and execution are carried out (3, 4). Virus-encoded Bcl-2 homologues effectively inhibit the intrinsic apoptotic pathway during virus infections, viz. adenovirus, Epstein-Barr virus, and herpesvirus (5–8). Viruses such as human papilomavirus, SV40, adenovirus, hepatitis B virus, human cytomegalovirus, and Epstein-Barr virus encode proteins that interact with p53 and disrupt its apoptosis-inducing properties (9–15). Another mechanism used by viruses to block apoptosis is to interfere with caspases. For example, p35 and p49 from baculovirus are potent inhibitors of most caspases (16–18). Orthopoxviruses contain a serpin, cytokine response modifier A (CrmA), that also directly inhibits several caspases (19). Similarly, the virus-encoded inhibitor of apoptosis (IAP) family of proteins, first discovered in baculoviruses, was shown to be involved in suppressing host cell death responses by regulating caspases. IAPs contain an N-terminal ~70-aa-long baculovirus IAP repeat domain and a C-terminal zinc-binding RING (really interesting new gene) finger domain.

The abbreviations used are: IAP, inhibitor of apoptosis; RelB, v-rel reticuloendotheliosis viral oncogene homologue B (NF-κB family protein); NF-κB, nuclear factor κB; Daxx, death domain-associated protein 6; M1, matrix 1 protein; cIAP, cellular IAP; Birc, baculovirus IAP repeat-containing protein; cFLIP, cellular FLICE-inhibitory protein; ND10, nuclear domain 10; PML, promyelocytic leukemia; Dnmt, DNA methyltransferase; CpG, cytosine phosphate guanine; DBM, Daxx binding motif; NLS, nuclear localizing signal; DAPK, death-associated protein kinase; p53, tumor suppressor; p53-kDa protein; DHB, Daxx helix bundle; h.p.i., hours postinfection; PR8, eighth strain of influenza A virus isolated from Puerto Rico; aa, amino acid(s); FLICE, Fas-associated death domain-like interleukin-1β-converting enzyme; m.o.i., multiplicity of infection; XIAP, X-linked inhibitor of apoptosis; hDaxx, human Daxx; Q-PCR, quantitative real time PCR; Ub, ubiquitin.

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**EXPERIMENTAL PROCEDURES**

**Viruses, Cells, and Viral Infection**—Madin-Darby canine kidney, human alveolar epithelial cell (A549), and 293T cells were grown in minimum Eagle's medium, Ham's F-12, and DMEM, respectively, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM l-glutamine, 2 mM sodium pyruvate, and 1× penicillin, streptomycin, and Fungizone at 37 °C with 5% CO2. For infection, cells were washed with phosphate-buffered saline (PBS) and infected with influenza A PR8 (A/Puerto Rico/8/34) strain at the indicated multiplicity of infection (m.o.i.) in PBS containing 0.2% bovine serum albumin (BSA), 1 mM MgCl2, 0.9 mM CaCl2, 100 units/ml penicillin, 0.1 mg/ml streptomycin for 45 min at 37 °C. The inoculum was aspirated, and A549 or Madin-Darby canine kidney cells were incubated in the respective medium supplemented with 0.2% BSA and antibiotics. The amount of infectious virus in cell supernatants was determined by plaque assay as described previously (57).

**Antibodies, Reagents, and Inhibitors**—Antibodies against M1 (sc-69824 and sc-17589), Daxx (sc-7152), RelB (sc-226), GFP (sc-8334), His (sc-803), cFLIP (sc-8347), and Dnmt3a (sc-20703) were from Santa Cruz Biotechnology (Santa Cruz, CA). β-Actin (551257), mouse double minute 2 (Mdm2) (556353)-, p53 (554294)-, phospho-p53 (558245), phosphoserine/threonine (612548)-, and Dnmt1 (612618)-specific antibodies were obtained from BD Biosciences. Antibodies against cleaved PARP (1065), cleaved GAPDH (12100), survivin (2808), XIAP (2045), phospho-PKCα (9375), and lamin A/C (2032) were from Cell Signaling Technology, Inc. (Danvers, MA). Anti-M2 (F3165) antibody was from Sigma-Aldrich. All antibodies were used at a 1:1000 dilution except anti-M1 and anti-β-actin, which were used at 1:500. Cycloheximide (Sigma, C7698) was used at 50 μg/ml, whereas MG132 (Sigma, C2211) was used at 20 μM/ml. Calphostin C (Sigma, C6303) was used at 80 nM.

**Plasmid and siRNA Transfection**—293T and A549 cells were either transfected with Lipofectamine 2000 (Invitrogen) or siPORT-NeoFX (Ambion, Austin, TX) according to the manufacturers’ instructions. Custom synthetic siRNA (5′-CTC CAG ATT TGC CTG AAG A-3′) against M1 was obtained from Dharmacco (Lafayette, CO). Control siRNA was from Qiagen (Hilden, Germany) (All Star Negative Control, 1027280).

**Western Blot Analysis**—Total protein was extracted with Totex buffer (20 mM HEPES at pH 7.9, 0.35 mM NaCl, 20% glycerol, 1% Nonidet P-40, 1 mM MgCl2, 0.5 mM EDTA, 1 mM EGTA, 50 mM NaF, and 0.3 mM NaVO3) containing a mixture of protease and phosphatase inhibitors (Sigma). Immunoblotting was performed with specific antibodies and visualized using an ECL Western blotting detection kit (Millipore, Billerica, MA).

**Cell Fractionation**—Cytosolic extracts free of nuclei and nuclear fractions were prepared. Briefly, cells were washed in ice-cold PBS, pH 7.2 and then in hypotonic extraction buffer (50 mM PIPES, pH 7.4,50 mM KCl, 5 mM EGTA, 2 mM MgCl2, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)) and centrifuged. The pellet was resuspended in hypotonic extraction buffer and lysed in a Dounce homogenizer. This cell lysate was centrifuged for 10 min at 750 × g at 4 °C to pellet nuclei, and the clarified cytosolic supernatant was either tested immediately or stored in aliquots at −80 °C. Nuclear
fractions were prepared by resuspending the pellet in ice-cold buffer C (10 mM HEPES, pH 7.9, 500 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% Nonidet P-40, 1 mM DTT, 1 mM PMSF, 8 mg/ml aprotinin, and 2 mg/ml leupeptin, pH 7.4) and kept for 30 min on ice with intermittent vortexing. The resuspended fraction was then spun at 14,000 × g for 30 min at 4 °C, and the supernatant (nuclear fraction) was stored in aliquots at −80 °C.

Co-immunoprecipitation—Cells were washed with ice-cold PBS and then lysed in a solution containing 10 mM Tris, pH 8.0, 170 mM NaCl, 0.5% Nonidet P-40, and protease inhibitors for 30 min on ice with three subsequent freeze/thaw cycles at −80 °C to lyse nuclei. Cell debris was removed by centrifugation, and the supernatants were precleared with protein A-coupled Sepharose beads for 2 h. The lysates were then immunoprecipitated with the indicated antibodies and isotype-matched control antibodies plus protein A-Sepharose for at least 4 h or overnight. Beads were washed four times with 1 ml of wash buffer (200 mM Tris at pH 8.0, 100 mM NaCl, and 0.5% Nonidet P-40) and once with ice-cold PBS and boiled in 2× loading buffer. Proteins were resolved by SDS-PAGE before probing with the indicated antibodies.

Quantitative Real Time PCR—Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer’s instructions. cDNA was prepared from 1–2 μg of RNA using SuperScript III reverse transcriptase (Invitrogen) with random hexamer primers. Real-time PCR reactions (50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s, and 72 °C for 10 min) were performed in triplicates using SYBR Green (Applied Biosystems, Foster City, CA) using GAPDH as a control. Primer sequences are available upon request.

 Luciferase Assays—293T cells were transfected with various plasmids using Lipofectamine 2000 reagent (Invitrogen) in 6-well plates and 4 μg of DNA/well. Cells were incubated for 30 h posttransfection, and luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer’s protocol. Firefly luciferase values were normalized to Renilla luciferase values. All experiments were done three or more times.

Confocal Microscopy and Imaging—To assess subcellular localization and interaction of M1, A549 cells cultured on coverslips were washed twice with cold PBS. Cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.2% Triton X-100 for 10 min, blocked with 5% BSA, and processed and incubated with primary antibodies against Daxx and M1 for 2 h at room temperature. Unbound primary antibodies were washed with PBS (three times) followed by a 1-h incubation (room temperature) with rhodamine- and DyLight 488-conjugated secondary antibody, respectively. After washing five times in PBS and mounting with VECTASHIELD (Vector Laboratories, Burlingame, CA), confocal microscopy was performed utilizing an LSM510-Meta confocal microscopy system (Zeiss) equipped with an argon ion laser (for DyLight 488 and GFP excitation with the 488 nm line), a helium-neon laser (for rhodamine excitation with the 543 nm line), and a Chameleon titanium-doped sapphire laser (for DAPI excitation with the 350 nm line). A 63× 1.4 numerical aperture oil immersion objective was used for all imaging. For comparisons between multiple samples, images were collected during a single session using identical excitation and detection settings. The detector gain settings were chosen to allow imaging of the desired cells within the linear range of the photomultiplier tube without saturating pixels. For imaging localization of different proteins, randomly chosen fields of cells were imaged with the above laser lines. The complete set of experiments was performed twice to eliminate artifacts arising from individual experiments.

Chromatin Immunoprecipitation Assays—M1-expressing 293T cells were subjected to formaldehyde cross-linking. Cross-linking reactions were quenched by PBS containing 0.125 mM glycine. Chromatin samples were sonicated to average length of 500 bp for immunoprecipitation; chromatin from 3 × 10⁶ cells was adjusted to a 1.0-ml volume with radiolabeled immunoprecipitation assay buffer and precleared with protein A-Sepharose for 30 min at 4 °C. Daxx was immunoprecipitated overnight with a rabbit polyclonal antibody. Following immunoprecipitation and reversal of cross-links, the samples were analyzed by PCR amplification using primers specific for the cIAP1 promoter (5′-CAT CCT GGC CTC CCA GC-3′ and 5′-CGC TCG GCT CGG ATC AG-3′) and cIAP2 promoter (5′-GTA GCC TGG AGA AGT TGA CCT ACC-3′). Control amplifications were accomplished using primers specific for the cyclophilin A gene (5′-CTCCCTTTGAGCTGTTTGCAG-3′ and 5′-CACCACATGCTTGCCATCC-3′). Amplifications were done using the following variables: 95 °C/4-min hot start followed by 26 cycles of 95 °C/45 s, 60 °C/30 s, and 72 °C/1 min. PCR products were analyzed in 2% agarose gels and stained with ethidium bromide followed by UV visualization.

Bisulfite Conversion and Genomic Sequencing—The methylation status of various promoters was assessed by bisulfite genomic sequencing. Briefly, genomic DNA was isolated from A549 and 293T cells by a high salt method (58). Bisulfite reactions were performed using an EpiTect bisulfite kit (59104, Qiagen) under conditions that allowed for complete conversion of cytosines, but not 5-methylcytosines, to uracil. The bisulfite-modified DNA was amplified by PCR using the following conditions: 2 min at 94 °C; 40 cycles of 30 s at 94 °C, 30 s at 60 °C, and 1.5 min at 68 °C; and finally 10 min at 68 °C. Amplified products were subcloned into the pCR2.1 TOPO vector via TA cloning (Invitrogen). Ten clones were sequenced (per target promoter). The methylation profile of the promoter of interest was determined by comparing the sequence of bisulfite-converted DNA with that of unmodified DNA.

In Vitro Association of Recombinant M1 with Daxx—Using the TnT Quick Coupled Transcription/Translation System (L1170, Promega, Madison, WI) in vitro translated native M1, M1(1–112), M1(95–252), ΔDBM-M1, and Daxx binding motif (DBM) point mutants of M1 protein (~5 μg) were immobilized on Ni²⁺ by overnight incubation in HEPES-buffered saline at 4 °C. After extensive washing in PBS with 0.3% Tween to remove unbound protein, M1 proteins were incubated with in vitro translated and enterokinase-treated human Daxx (hDaxx) (10 μg) for 4 h at 4 °C in HEPES-buffered saline. Beads were washed extensively with HEPES-buffered saline to remove non-specifically bound proteins. Remaining proteins were separated by 4× sample buffer, then analyzed by SDS-PAGE, and immunoblotted for Daxx.
**In Vivo Ubiquitination**—293T cells were treated with MG132 for 3–6 h after the cells were transfected or not with pcD-M1 and pcDNA6-UbB and then lysed in 1% SDS. After boiling for 5 min, lysates were diluted 10 times with cold lysis buffer supplemented with 1× Complete inhibitor and 10 mM N-ethylmaleimide (Sigma). After immunoprecipitation with the indicated antibodies, the immunoprecipitates were resolved by 7% SDS-PAGE and transferred onto nitrocellulose membrane. The blot was blocked in 5% BSA and probed with an anti-ubiquitin antibody (Pierce) or anti-His antibody according to the manufacturers’ instruction.

**Statistical Analysis**—Data are expressed as mean ± S.D. of at least three independent experiments (n ≥ 3). In all tests, p = 0.05 was considered statistically significant.

**RESULTS**

**Involvement of M1 in Transcription Regulation of Antia apoptotic Genes**—It has been reported previously that M1 localizes to the nucleus early during infection (55). In this study also, nuclear accumulation of M1 from 2 h.p.i. was observed (Fig. 1A). However, after 8 h.p.i., M1 protein levels decreased in the nucleus, and it accumulated in the cytoplasm (Fig. 1A). Further-
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more, confocal laser-scanning imaging also confirmed nuclear accumulation of M1 protein during early time points (2 and 4 h.p.i.) of PR8 infection (Fig. 1B). M1 protein has been shown to associate with PML nuclear body proteins (51, 52), and Daxx has been shown to repress RelB (38, 39). Thus, during early infection of influenza A (2–8 h.p.i.), expression of NF-κB (RelB) regulated apoptosis-modulating genes including cIAP1 (Birc2), cIAP2 (Birc3), survivin (Birc5), and cFLIP was analyzed. The expression of candidate target genes was analyzed by quantitative real-time PCR (Q-PCR) after A549 cells were infected with influenza A PR8 strain (A/Puerto Rico/8/34) at 1 m.o.i. for the given postinfection times. We observed ~6-fold higher levels of cIAP1 (Birc2) and cIAP2 (Birc3) mRNA, ~2-fold higher survivin (Birc5) mRNA, ~4-fold higher cFLIP (cFLAR) mRNA, and ~2-fold higher XIAP mRNA levels in infected cells at 4 h.p.i. compared with control cells (Fig. 1C). In contrast, TRAF6 and GAPDH mRNA levels were similar in uninfected and infected A549 cells (Fig. 1C). Protein analysis by immunoblotting corroborated mRNA expression data, showing increased cFLIP, cIAP1, cIAP2, survivin, and XIAP levels but not β-actin (Fig. 1D). A significant increase in protein levels can be seen from 2 to 4 h.p.i. The expression pattern of influenza A viral M1 was also analyzed by immunoblotting, and it was consistent with its transcript level (supplemental Fig. S1). Because most of these genes are antiapoptotic, the role of M1 protein in their up-regulation was assessed by using M1-specific siRNA. A549 human lung epithelial cells were transiently transfected with either scrambled siRNA (60 nmol) or M1 siRNA (60 nmol) or left untreated for 24 h followed by infection with influenza A PR8 strain (A/Puerto Rico/8/34) at 1 m.o.i. for 4 h. In M1 siRNA-treated cells, cIAP1 transcripts were ~3-fold less, cIAP2 transcripts were ~4-fold less, cFLIP transcripts were ~4.5-fold less, and XIAP transcripts were ~4.5-fold less compared with only PR8-infected or scrambled siRNA-treated cells (Fig. 1E). This result was further confirmed by immunoblotting whole cell lysates from scrambled siRNA-treated (60 nmol) or M1 siRNA-treated (60 nmol) and/or untreated PR8-infected cells. Consistent with the previous result, significant decreases in protein levels of these antiapoptotic genes (cIAP1, cIAP2, and cFLIP) were observed in the M1 siRNA-treated cells but not in scrambled siRNA-treated or PR8-infected cells (Fig. 1F). Effective down-regulation of M1 gene by M1 siRNA was confirmed by immunoblot analysis along with scrambled siRNA (Fig. 1G). Influenza A nucleoprotein was also measured as control. Thus, these results together suggest that expression of M1 protein may play a role in transcriptional up-regulation of antiapoptotic genes during early infection periods.

M1 Prevents Daxx from Binding RelB—Because during infection RelB-regulated antiapoptotic genes that are normally inhibited by the protein Daxx were up-regulated, we hypothesized that RelB and Daxx association might be prevented by influenza A virus. Thus, a co-immunoprecipitation experiment was performed to analyze RelB and Daxx association during PR8 infection. Whole cell lysates prepared after A549 cells were treated with scrambled siRNA or M1 siRNA (60 nmol) and/or infected with PR8 (2, 4, 6, and 8 h.p.i.) were immunoprecipitated with an anti-Daxx polyclonal antibody followed by immunoblotting with RelB antibody. In M1 siRNA-treated cells and in infected cells (0–2 h.p.i.), RelB co-immunoprecipitated with Daxx, whereas weak or no co-immunoprecipitation was observed from 4 h.p.i. onward in only PR8-infected or in scrambled siRNA-treated PR8-infected cells (Fig. 2A). Because our previous experiment suggested a role for M1 protein in transcription regulation, RelB and Daxx association was assessed in the presence of M1 alone. To perform this experiment, 293T cells were transfected with pcDNA6-M1 (pcD-M1) or pcDNA6 plasmid (control) alone and left untreated for 36 h before whole cell lysates were immunoprecipitated with an anti-Daxx antibody followed by immunoblotting with RelB and M1 antibodies. Result showed poor association between RelB and Daxx in M1-expressing cells compared with control cells (Fig. 2B), whereas a large amount of M1 protein precipitated with Daxx (Fig. 2B, lane 3). In a reciprocal immunoprecipitation using RelB antibody, Daxx co-immunoprecipitated with RelB in both M1-expressing and control cells (Fig. 2C); however, RelB and Daxx interaction was significantly reduced (>3.0-fold) in M1-expressing cells (Fig. 2C, lane 3), confirming that M1 protein expression alone results in reduction of Daxx-RelB complex formation even in the absence of Influenza A virus infection (Fig. 2C). This was further confirmed by confocal microscopy studies (supplemental Fig. S2). As overexpressing M1 prevents Daxx from binding RelB (Fig. 2, B and C) and M1 siRNA effectively reduced transcription of the antiapoptotic genes, we assumed that M1 may be directly associated with any of the partners of Daxx-RelB complex. To analyze this possible interaction of Daxx or RelB with M1, whole cell lysates after PR8 infection were immunoprecipitated with an anti-M1 polyclonal antibody followed by immunoblotting with Daxx or RelB antibody. As shown in Fig. 2D, M1 protein immunoprecipitated with endogenous Daxx but not with RelB (Fig. 2D and supplemental Fig. S2). Furthermore, the co-association was confirmed by reciprocal immunoprecipitation by Daxx antibody followed by immunoblotting with M1 and RelB antibodies, taking lysates from PR8-infected and mock-infected cells 4 h postinfection (Fig. 2E). This was further confirmed by immunoprecipitating cell lysates with an anti-FLAG antibody that was immunoblotted with Daxx or RelB antibody after 293T cells were transfected with pFLAG-CMV6-M1 or pFLAG-CMV6 (Fig. 2F). In this experiment, RelB served as a negative control. Confocal laser-scanning microscopy of A549 cells after PR8 infection (4 h.p.i.) and 293T cells after pEGFP-c2-M1 transfection (24 h) confirmed co-localization of cellular Daxx and influenza viral M1 protein (Fig. 2G). These results collectively show that M1 protein directly interacts with the cellular protein Daxx, and this binding disrupts Daxx-RelB association during influenza A infection.

M1 Alone Can Induce Survival Genes by Impairing Promoter Binding of Daxx—To analyze whether M1 alone in the absence of other viral proteins can induce the RelB-dependent survival genes, 293T cells were either mock-transfected or transfected with scrambled siRNA or M1 siRNA (60 nmol) and left untreated for 24 h followed by transfection with pcD-M1. After 32 h, whole cell lysates were prepared for immunoblotting. Results revealed that in cells overexpressing M1 alone or in the presence of scrambled siRNA RelB-dependent genes (cIAP1, cIAP2, cFLIP, and XIAP) were up-regulated compared with M1
siRNA-treated cells (Fig. 3A, lane 4) or non-transfected control (Fig. 3A, lane 1). Furthermore, the transcript levels of these genes were analyzed in pcD-M1 transiently transfected cells where ~44-fold higher cIAP1 mRNA, ~35-fold higher cIAP2 mRNA, ~42-fold higher cFLIP mRNA, ~16-fold higher XIAP mRNA, and ~10-fold higher survivin mRNA were observed (Fig. 3B). However, the TRAF6 transcript level was unchanged (Fig. 3B). In cells where hDaxx was overexpressed following transfection with pcD-hDaxx, reduced expression of cFLIP and IAP genes was observed (Fig. 3C), whereas when pcD-hDaxx and pcD-M1 were co-transfected, an increase in transcripts of cIAP1, cIAP2, cFLIP, XIAP, and survivin, but not in TRAF6, was observed (Fig. 3C). This suggested that M1 is a strong inhibitor of the transcription repressional activity of Daxx protein.

Next, we investigated whether M1 influences the ability of Daxx to repress promoters of target genes. We performed reporter gene assays using pcD-RelB, pcD-Daxx, and cIAP1, cIAP2, cFLIP, or Bcl-x promoter-luciferase reporter gene constructs. Transient transfection of reporter gene constructs together with pcD-hDaxx led to suppression of these promoters, but in the presence of M1 protein, the promoter activity was restored (Fig. 3D). No change was observed in Bcl-x promoter activity.

To explore whether M1 association with Daxx prevents the latter from binding RelB-responsive promoters, chromatin...
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immunoprecipitation (ChIP) followed by Q-PCR analysis was performed. ChIP assays during PR8 infection showed that association of Daxx with cIAP1, cIAP2, and cFLIP promoters in A549 cells was significantly reduced (Fig. 3E). Furthermore, a ChIP assay was carried out in 293T cells transfected with pcDNA6-RelB, pcDNA6-Daxx, Renilla luciferase reporter (pRL-TK), and either cIAP1-pGL3-basic, cIAP2-pGL3-basic, cFLIP-pGL3-basic, or BCL-X-pGL3 (cloned promoters) and/or pcD-M1. The ratio of experimental vector to co-reporter vector (Renilla luciferase) was 10:1. Luciferase assays were performed 24 h posttransfection. Bcl-x gene promoter served as a control. All assays were performed in triplicate. E and F, chromatin was immunoprecipitated using anti-Daxx antibody or control IgG antibodies to determine association of Daxx with RelB candidate target gene promoters (cIAP1, cIAP2, and cFLIP) after A549 cells were infected with PR8 (1 m.o.i.) strain for the given time points (2, 4, and 6 h.p.i.) or after 293T cells were transfected with pcD-M1 and left untreated for 36 h. Target gene promoters were amplified by Q-PCR (40 cycles) using promoter-specific primers that encompassed NF-κB binding sites. Values represent averages of three Q-PCRs from three ChIP experiments. G, 293T cells were transiently transfected with pcD-M1 and left untreated for 36 h before chromatin was precipitated using anti-Daxx antibody, and recovered promoters were amplified by Q-PCR (40 cycles) using primers for the cIAP1, cIAP2, and TRAF6 promoters and then analyzed by gel electrophoresis. TRAF6 gene promoter served as a control. Input represents 10% of the chromatin specimen directly analyzed by Q-PCR without immunoprecipitation. Error bars represent S.D.
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FIGURE 4. Methylation of target promoters decreases as M1 prevents Dnmts from binding Daxx. A, A549 cells were infected with PR8 (1 m.o.i.) strain for the given time points (0, 2, 4, 6, and 8 h.p.i.) before cell lysates were immunoprecipitated using anti-Daxx antibody followed by immunoblotting with Dnmt1 and Dnmt3a antibodies. IgG served as a loading control, and input lysate (PR8-infected cell lysate without immunoprecipitation (IP)) was used for antibody specificity. B, to analyze the role of M1 in disruption of Daxx-Dnmt association, A549 cells were transfected with scrambled siRNA or M1-specific siRNA (60 nmol) prior to PR8 infection (1 m.o.i., 4 h.p.i.), and cell lysates of infected (0 and 4 h.p.i.) and siRNA-treated infected (4 h.p.i.) cells were immunoprecipitated with anti-Daxx antibody followed by immunoblotting with Dnmt1 antibody. Cell lysate without immunoprecipitation was used as input. C, 293T cells were co-transfected with pFLAG-Daxx and pcD-M1 after the cells were transfected or not with M1 siRNA (60 nmol). After 24 h of transfection, the whole cell lysates were subjected to immunoprecipitation with antiFLAG antibody followed by immunoblotting with Dnmt1 and Dnmt3a antibodies. Only pFLAG-Daxx-transfected cell lysates served as a control, and lysate without immunoprecipitation served as input. D, 293T cells were transfected or not (Control) with pcD-M1 and left untreated for 36 h before the cell lysates were immunoprecipitated with anti-Dnmt1 antibody followed by immunoblotting with anti-Daxx antibody. IgG served as a loading control, and total cell lysate was used as input. E, to detect Dnmt1 complexed with RelB in the presence of M1, RelB was immunoprecipitated (36 h posttransfection) with anti-RelB antibody using control and M1-overexpressing (pcD-M1-transfected) 293T cell lysates followed by immunoblotting with anti-Dnmt1. F and G, genomic DNA from control and PR8-infected (1 m.o.i., 4 h.p.i.) A549 cells and control and pcD-M1-transfected 293T cells was isolated and treated with bisulfite, and 0.5 μg of genomic DNA was used for bisulfite conversion and subsequent PCR amplification. PCR products were cloned and sequenced. The methylation profile of the promoters was determined by comparing the sequence of bisulfite-converted DNA with unmodified DNA. The percentage of methylated CpG sites is shown for each promoter examined. The values represent a significance value of \( p \leq 0.0001 \). Error bars represent S.D.

cipitation using RelB antibody because Daxx-Dnmt1 complex interacts with RelB. Consistent with disruption of Dnmt1-Daxx interaction by M1 protein (Fig. 4D), M1 also prevented Dnmt1-RelB association (Fig. 4E) as M1 interaction with Daxx resulted in poor association of RelB and Dnmt1 with Daxx. As Dnmts function in Daxx-mediated repression of genes by modulating methylation of promoters, we investigated the methylation status of the RelB target promoters, viz. cIAP1, cIAP2, and cFLIP, during PR8 infection. Bisulfite treatment and sequencing of promoters were performed and showed extensive methylation differences in the target gene promoters between control and PR8-infected A549 cells (supplemental Fig. S3). With a significant decrease in promoter methylation, the percentage of CpG dinucleotide methylation for cIAP1 was ~2.8% in infected cells compared with ~14% in control cells, for cIAP2 was ~2% in infected cells versus ~8% in control cells, and for cFLIP was ~0.8% in infected cells versus ~5% in control cells (Fig. 4F). In addition, only ~1.8% (cIAP1 promoter), ~2% (cIAP2 promoter), and ~0.3% (cFLIP promoter) CpG dinucleotide methylation was observed in M1-overexpressing cells compared with ~12% (cIAP1), ~10% (cIAP2), and ~4.5% (cFLIP) methylation in control cells (Fig. 4G). Overall, the data suggest that M1 binds to Daxx, resulting in reduced Daxx-Dnmt1 interaction, which leads to reduced Dnmt1-RelB interaction. This results in decreased methylation and transcriptional up-regulation of RelB-responsive antiapoptotic genes.

Critical Role of N-terminal DBM in Daxx Binding—The secondary structure of M1 protein is shown (Fig. 5A). Deletion mutants pFLAG(1–112)M1 and pFLAG(95–252)M1 were transfected in 293T cells along with wild-type M1 (pFLAG-M1) followed by immunoprecipitation with anti-Daxx antibody to identify the Daxx binding region. Results showed that compared with wild-type M1 protein a similar interaction level was observed between the mutant M1(1–112) and Daxx, whereas significantly reduced interaction was observed between M1(95–252) and Daxx (Fig. 5B). This suggests that the N-terminal region is primarily involved in Daxx binding. Substrates of Daxx generally bind to its Daxx helix bundle (DHB) domain with a specific DHB domain-interacting motif (59). A putative DHB-interacting motif or DBM was identified at aa 23–48 region of the N terminus of M1 protein (Fig. 5C, colored). Furthermore, deletion mutants (pFLAG(49–252)M1 and
pFLAG(ΔDBM)M1) and substitution mutants (core-forming amino acids of DBM; Leu, Trp, and Glu) (pFLAG(L42R/W45S)M1 and pFLAG(E40A/W45S)M1) of the DBM of M1 were constructed (Fig. 5D). Daxx association was assessed by immunoprecipitation of cell lysates with anti-FLAG antibody after 293T cells were transiently transfected with pFLAG-M1, pFLAG(ΔDBM)M1, and pFLAG(E40A/W45S)M1. As shown in Fig. 5E, both the deletion and point mutants bound to Daxx with significantly less affinity compared with wild-type M1. For further confirmation, in vitro binding experiments were performed with the in vitro translated hDaxx (pcD-hDaxx), wild-type M1, and M1 mutant proteins (pcDNA constructs). Native M1, M1(1–112), ΔDBM-M1, (L42R/W45S)M1, and (E40A/W45S)M1 proteins were immobilized on nickel beads and then incubated overnight at 4 °C with translated Daxx protein. Beads were washed with HEPES-buffered saline, and proteins were subjected to gel electrophoresis, transferred, and immunoblotted with anti-Daxx antibody. G, expression of wild-type and mutant M1 proteins was confirmed by immunoblotting after 293T cells were transiently transfected with the respective M1 constructs. H, to directly analyze the antiapoptotic role of M1, control along with wild-type and mutant M1 construct (pcD-M1, pcD(1–112)M1, pcD(ΔDBM)M1, pcD(L42R/W45S)M1, and pcD(E40A/W45S)M1)-transfected 293T cells were mock or control UV-C (254 nm)-irradiated at a dose of 50 J/m² for 15 min. The dish covers were removed during irradiation, and after treatment, covers were replaced, and then cells were incubated at 37 °C for 4 h. The cells were lysed, and the whole lysates were subjected to SDS-PAGE followed by immunoblotting with anti-caspase-8 and anti-caspase-3 antibody. β-Actin served as a loading control.

**FIGURE 5.** M1 binds Daxx through DBM. A, secondary structure of M1 showing it has an NLS (aa 101–105), membrane binding domain (aa 1–164), and ribonucleoprotein (RNP)-binding region (aa 164–252). Schematic drawings of the deletion mutants M1(1–112) and M1(95–252) are presented. Both mutants independently can localize to the nucleus because of the presence of the NLS. B, 293T cells were transiently transfected with pFLAG-M1, pFLAG(ΔDBM)M1, and pFLAG(E40A/W45S)M1 constructs and left untreated for 36 h before immunoprecipitation (IP) was performed with anti-FLAG antibody followed by immunoblotting with anti-Daxx antibody. IgG served as a loading control, and pFLAG-M1-transfected whole cell lysate served as input. C, general letter codes of the entire (252-aa) native amino acid sequence of M1. The putative DBM (aa 23–48) is presented in the amino acid sequence of M1. The DBM is characterized by five negatively charged (Glu-23, Glu-29, Asp-30, Asp-38, and Glu-40; red), two aliphatic (Val-41 and Leu-42; yellow), one polar (Glu-44; red), one aromatic (Trp-45; green), and two final polar (Lys-47 and Thr-48; pink) residues. D, schematic drawings of the deletion mutants for DBM used in the binding study are presented. E, immunoprecipitation with anti-FLAG antibody was performed after 293T cells were transfected with pFLAG-M1, pFLAG(ΔDBM)M1, and two point mutants of DBM, pFLAG(ΔDBM)M1, and pFLAG(E40A/W45S)M1, followed by immunoblotting with anti-Daxx antibody. IgG served as a loading control. F, an in vitro binding experiment was performed after hDaxx (pcD-Daxx), M1, and M1 mutant proteins were in vitro translated from their pcDNA constructs (pcD-M1, pcD(1–112)M1, pcD(95–252)M1, pcD(ΔDBM)M1, pcD(L42R/W45S)M1, and pcD(E40A/W45S)M1). His-tagged M1 and M1 mutant proteins were first nickel-immobilized and then incubated overnight at 4 °C with translated Daxx protein. Beads were washed with HEPES-buffered saline, and proteins were subjected to gel electrophoresis, transferred, and immunoblotted with anti-Daxx antibody. G, expression of wild-type and mutant M1 proteins was confirmed by immunoblotting after 293T cells were transiently transfected with the respective M1 constructs. H, to directly analyze the antiapoptotic role of M1, control along with wild-type and mutant M1 construct (pcD-M1, pcD(1–112)M1, pcD(ΔDBM)M1, pcD(L42R/W45S)M1, and pcD(E40A/W45S)M1)-transfected 293T cells were mock or control UV-C (254 nm)-irradiated at a dose of 50 J/m² for 15 min. The dish covers were removed during irradiation, and after treatment, covers were replaced, and then cells were incubated at 37 °C for 4 h. The cells were lysed, and the whole lysates were subjected to SDS-PAGE followed by immunoblotting with anti-caspase-8 and anti-caspase-3 antibody. β-Actin served as a loading control.
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Pulldown experiments (Fig. 5F). These results confirmed that the N-terminal DBM of M1 is critical for its interaction with Daxx; however, other regions may have role in binding. To prove direct functional involvement of M1 in inhibiting apoptosis, 293T cells were transfected with wild-type M1 (pcD-M1) and its deletion (pcD(1–112)M1) and pcD(ΔDBM)M1) and substitution (generated through point mutation) mutants (pcD(L42R/W45S)M1 and pcD(E40A/W45S)M1). Transfected and non-transfected cells were UV-irradiated for 15 min and left untreated for 4 h followed by immunoblotting of the cell lysates for activation of caspase-8 and caspase-3. Results showed that upon UV irradiation pcD-M1- and pcD(1–112)M1-transfected cells showed significantly reduced caspase-8 and caspase-3 activation than the non-transfected positive control cells or the pcD(ΔDBM)M1-, pcD(L42R+W45S)M1-, and pcD(E40A+W45S)M1-expressing cells (Fig. 5F). A fluorometric assay for caspase activation was also done, and it supports the immunoblot data (data not shown). This experiment proves that M1 is directly involved in inhibiting apoptosis, which is dependent on its Daxx binding ability.

Phosphorylation-mediated Translocation of M1 to Nucleus—Previous results confirmed the survival function of M1 during early stages of PR8 infection. During early infection (2–8 h.p.i.), M1 protein predominantly resides within the nucleus (Fig. 1, A and B) and interacts with nuclear Daxx, but after 8 h.p.i., M1 accumulates mainly in the cytoplasm (55). Hence, it can be postulated that the survival function of M1 primarily depends on its translocation to nucleus. Therefore, we wanted to solve the enigma of what drove the translocation of M1 to the nucleus, and we assumed that structural changes like phosphorylation or any other modification might be involved. M1 has been shown to be phosphorylated at aa 53, 70, 161, and 185 (53, 55) by cellular kinases (Fig. 6A). Thus, Ser to Ala substitution mutants were constructed, namely pcD-ΔNLS-M1, pcD(S53A)M1, pcD(S70A)M1, pcD(S161A)M1, and pcD(S185A)M1. 293T cells were transiently transfected with wild-type M1 and mutant M1 constructs followed by cellular fractionation (nucleus and cytoplasm). Immunoblotting revealed that only wild-type M1 was present in both the nucleus and cytoplasm, whereas mutant proteins along with NLS-deleted protein were present predominantly in the cytoplasm with negligible presence in nucleus (Fig. 6B). This suggested that both M1 phosphorylation and the NLS motif were necessary for its translocation to the nucleus (Fig. 6B). Confocal imaging of A549 cells transfected with pEGFPc2 constructs of wild-type M1 and its NLS deletion mutant and phosphomutants confirmed the previous result that M1 phosphorylation was essential for its nuclear translocation (Fig. 6C). In a reciprocal experiment, A549 cells were either treated with calphostin C (100 nM), a potent protein kinase C inhibitor, or left untreated before the cells were infected with PR8 (0, 4, and 6 h.p.i.). Subsequently, immunoprecipitation was done from nuclear lysates with anti-phosphoserine antibody and then immunoblotted with M1 antibody. Results showed that in the presence of calphostin C there was significantly less M1 protein in the nucleus compared with PR8-infected control cells (Fig. 6F). This suggests that reduced phosphorylation may affect M1 translocation to the nucleus. Phospho-PKCα levels during PR8 infection also correlated with the M1 phosphorylation (Fig. 6E). Confocal imaging also proved that in the presence of calphostin C (100 nM) during early periods of PR8 infection M1 did not accumulate in the nucleus as it did in untreated PR8-infected cells (Fig. 6F). These results together suggest that the nuclear translocation of M1 was cellular kinase-mediated and phosphorylation-dependent.

M1 Controls the Stability of Daxx—During PR8 infection, Daxx protein levels were up-regulated compared with uninfected cells (Fig. 7A). In addition, 293T cells were transfected with pcD-M1, and the Daxx level was analyzed by immunoblotting. An almost 2-fold increase in Daxx protein level was observed in M1-overexpressing cells (Fig. 7B). Whether Daxx up-regulation is due to an increase in transcription or increased stability of the protein was examined by pulse-chase experiment. 293T cells were transfected or not with pcD-M1 followed by treatment with 50 μg/ml cycloheximide for the given time points. Immunoblot analysis revealed that the half-life of Daxx was increased in M1-overexpressing cells compared with control (Fig. 7C). The role of M1 in stabilizing Daxx was further confirmed as significant reduced stability of Daxx was observed in PR8-infected cells in the presence of M1 siRNA (60 nmol) compared with only PR8-infected or scrambled siRNA-transfected cells in the presence of cycloheximide (50 μg/ml) (Fig. 7D). To assess whether M1 modulates ubiquitination of Daxx to increase its stability, M1-overexpressing cells were treated with 20 μM MG132 for 3 h, and cell lysates were immunoprecipitated with anti-Daxx antibody to detect associated endogenous ubiquitin (Ub). In the presence of M1, less ubiquitin associated with Daxx (Fig. 7E). This was also confirmed by co-transfecting pFLAG-M1 with pcDNA6-UbB where less interaction between Daxx and UbB was observed in the presence of M1 and UbB proteins compared with only UbB-overexpressing cells (Fig. 7F). To assess the role of nuclear translocation of M1 in Daxx stability, 293T cells were transfected with either pcD-M1 or serine mutants of M1 (pcD(S53A)M1, pcD(S70A)M1, pcD(S161A)M1, and pcD(S185A)M1) followed by treatment with cycloheximide (50 μg/ml). As shown in Fig. 7G, increased stability of Daxx was observed in wild-type M1-expressing cells. Overall, the results confirmed that M1 regulates Daxx stability by preventing its ubiquitination and proteasomal degradation.

DISCUSSION

Cellular intrinsic immunity such as apoptosis and transcriptional repression generally contribute to restrict viral infection. However, viruses either encode certain proteins or modulate cellular proteins to evade transcription repression and apoptosis (5–19). PML nuclear bodies and their associated proteins such as PML, Sp100 (ND10-associated speckled, 100 kDa), Daxx, and ATRX (α thalassemia/mental retardation syndrome X-linked) as cellular defenses against viruses have been studied extensively (42–50). PML and Daxx associate with histone deacetylases, resulting in transcriptional repression (60), and Daxx recruits Dnmts to methylate and silence specific genes (38, 39). Viral proteins of different viruses such as human cytomegalovirus, adenovirus, Epstein–Barr virus, human papillomavirus, avian sarcoma virus, Puumala virus, dengue virus, and
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Phage φC31 virus have been shown to interact with Daxx, resulting in either degradation or reorganization of Daxx to counteract its antiviral function for efficient viral replication (42, 48). We show here that M1, the major structural protein of influenza A virus, interacts with Daxx (Fig. 2, B, D, E, F, and G) and disrupts its ability to form a complex with RelB (Fig. 2, A, B, and C). The impact of this interaction is the activation of RelB-regulated, Daxx-repressed genes including IAPs (cIAP1, cIAP2, and survivin) and Flip genes (Fig. 3, A, B, and C). However, in contrast, death-associated protein kinases (mainly DAPK1 and DAPK3) were also activated (supplemental Fig. S4), which is consistent with the previous report that in several human tumor cell lines Daxx controls DAPK gene expression (61).

Although this interaction activated apoptosis-regulating kinases, the overall effect of this association is prosurvival as some of the activated survival genes tend to counterbalance the effect of DAPKs (20–23). In response, influenza A virus-infected cells produce a number of cytokines (62). Although RelB is known to be stimulated by cytokines (63), no such significant increase in the transcript level of RelB was observed following influenza A infection (supplemental Fig. S1).

Daxx lacks domains for sequence-dependent DNA binding (37), and it binds promoters through physical interaction with RelB (38, 39), but during influenza A infection or in M1-overexpressing cells, promoter binding of Daxx was repressed (Fig. 3, G). In M1 siRNA-treated cells during PR8 infection, Daxx interacted with Dnmt1 and Dnmt3a (Fig. 4, A and B), which is consistent with the previous observation (39); however,
M1-Daxx interaction prevented binding of Dnmts with Daxx (Fig. 4, B, C, and D). Daxx recruitment of Dnmts results in DNA methylation of cIAP1, cIAP2, cFLIP, and survivin gene promoters that typically involves methylation of certain CpGs (39). As expected, the percentage of CpG methylation was significantly reduced in the presence of M1 protein (Fig. 4, F and G). On the basis of function, M1 has a membrane binding domain (aa 1–164), ribonucleoprotein binding domain (aa 165–252), and NLS (aa 101–105) (Fig. 5A). A deletion mutation study of M1 revealed that the N-terminal region binds significantly with Daxx protein similarly to full-length M1, whereas a low level of Daxx binding was observed with the C terminus (Fig. 5B). Hence, the full-length M1 protein is more efficient in binding Daxx, and this is expected because the binding partner, cellular Daxx, is a very large protein of 744 amino acids and has several domains such as DHB, helical, acidic, SPE (Ser/Pro/Glu), and SPT (Ser/Pro/Thr) and motifs such as small ubiquitin-like modifier interaction motifs (59). However, the N-terminal DHB domain attributes most of the substrate binding and determines the physicochemical properties of the entire molecule (59). Although the molecule is positively charged, several positively charged and negatively charged residues cluster on opposite sides of the molecule. In addition, an exposed hydrophobic patch along with positively charged residues is present on the surface of Daxx (59). These features of Daxx are key to the vast range of interacting partners.

The DHB domain interaction motif is characterized by the presence of four negatively charged residues (glutamic acid or aspartic acid) followed by an aliphatic, two polar, two aromatic, and a final polar residue (59). A somewhat similar motif was observed at the N-terminal DBM of M1 that is characterized by five negatively charged residues (Glu-23, Glu-29, Asp-30, Asp-38, and Glu-40), two aliphatic (Val-41 and Leu-42), one polar (Glu-44), one aromatic (Trp-45), and two final polar (Lys-47, 48) residues.

FIGURE 7. M1 increases Daxx stability. A, A549 cells were infected with PR8 (1 m.o.i.) for the given time points (0, 2, 4, 6, and 8 h.p.i.) followed by immunoblotting of the cell lysates with anti-Daxx antibody. β-Actin served as a loading control. B, expression of Daxx protein was analyzed after 293T cells were transiently transfected with pcD-M1, and after 32 h, cell lysates were electrophoresed and immunoblotted with anti-Daxx antibody. β-Actin served as a loading control. C, pulse-chase analysis for Daxx was performed after 293T cells were transfected or not with pcD-M1 and left untreated for 26 h before treatment with cycloheximide (50 μg/ml) for the given time points (0, 2, 4, and 6 h) followed by immunoblotting of the cell lysates with anti-Daxx antibody. β-Actin served as a loading control. D, stability of Daxx during PR8 infection (1 m.o.i.; 0, 2, 4, 6, and 8 h.p.i.) was assessed by pulse-chase experiment in A549 cells transfected or not with scrambled and M1 siRNAs (60 nmol). During infection, cells were treated with cycloheximide (CHX; 50 μg/ml), and then cell lysates were subjected to immunoblotting with anti-Daxx antibody. β-Actin served as a loading control. E, in vivo ubiquitination was performed for Daxx. 293T cells were transfected or not with pcD-M1 and after 32 h treated with MG132 (20 μM) for 3 h followed by immunoprecipitation with anti-Daxx antibody and immunoblotting with anti-ubiquitin antibody to detect ubiquinated Daxx. IG served as a loading control. F, 293T cells were transfected with pcFLAG-M1 and/or with pcD-UbB and left untreated for 32 h followed by MG132 (20 μM) treatment for 3 h and immunoprecipitation with anti-Daxx antibody. Ubiquitinated Daxx was detected by immunoblotting the pulled down proteins with anti-His antibody. IG served as a loading control. G, role of M1 point mutants in Daxx stability was assessed. 293T cells were transfected or not (Control) with wild-type and point mutants of M1 (pcD-M1, pcD(S35A)M1, pcD(S70A)M1, pcD(S161A), and pcD(S185A)M1) and after 32 h treated with cycloheximide (50 μg/ml) for 6 h. Cell lysates were subjected to immunoblotting with anti-Daxx antibody. β-Actin served as a loading control.

M1-Daxx interaction prevented binding of Dnmts with Daxx (Fig. 4, B, C, and D). Daxx recruitment of Dnmts results in DNA methylation of cIAP1, cIAP2, cFLIP, and survivin gene promoters that typically involves methylation of certain CpGs (39). As expected, the percentage of CpG methylation was significantly reduced in the presence of M1 protein (Fig. 4, F and G). On the basis of function, M1 has a membrane binding domain (aa 1–164), ribonucleoprotein binding domain (aa 165–252), and NLS (aa 101–105) (Fig. 5A). A deletion mutation study of M1 revealed that the N-terminal region binds significantly with Daxx protein similarly to full-length M1, whereas a low level of Daxx binding was observed with the C terminus (Fig. 5B). Hence, the full-length M1 protein is more efficient in binding Daxx, and this is expected because the binding partner, cellular Daxx, is a very large protein of 744 amino acids and has several domains such as DHB, helical, acidic, SPE (Ser/Pro/Glu), and SPT (Ser/Pro/Thr) and motifs such as small ubiquitin-like modifier interaction motifs (59). However, the N-terminal DHB domain attributes most of the substrate binding and determines the physicochemical properties of the entire molecule (59). Although the molecule is positively charged, several positively charged and negatively charged residues cluster on opposite sides of the molecule. In addition, an exposed hydrophobic patch along with positively charged residues is present on the surface of Daxx (59). These features of Daxx are key to the vast range of interacting partners.

The DHB domain interaction motif is characterized by the presence of four negatively charged residues (glutamic acid or aspartic acid) followed by an aliphatic, two polar, two aromatic, and a final polar residue (59). A somewhat similar motif was observed at the N-terminal DBM of M1 that is characterized by five negatively charged residues (Glu-23, Glu-29, Asp-30, Asp-38, and Glu-40), two aliphatic (Val-41 and Leu-42), one polar (Glu-44), one aromatic (Trp-45), and two final polar (Lys-47, 48) residues.
and Thr-48) residues (Fig. 5C). The DBM of M1 is critical because its functional binding to Daxx was confirmed when mutations in this motif resulted in poor binding (Fig. 5E). A deletion mutation or point mutations such as substitution of the core-forming residues tryptophan, leucine, and glutamic acid to serine, arginine, and alanine, respectively, abrogated Daxx binding (Fig. 5, E and F). Furthermore, an in vitro binding assay also confirmed the significance of DBM in binding cellular Daxx (Fig. 5F). These DBM mutants also failed to inhibit caspase (caspase-8 and -3) activation when apoptosis was induced by UV irradiation, whereas wild-type M1 or an N-terminal fragment of M1 (aa 1–112) with the DBM motif prevented caspase activation significantly (Fig. 5F). Although M1 mutants showed some protection against UV irradiation, it was less than wild type or the N-terminal fragment but more than the UV-treated positive control cells. Interestingly, the N-terminal part was more efficient in preventing caspase activation than was full-length M1 protein. This experiment proved an antiapoptotic role for M1 protein.

Phosphorylation is one of the known modifications of M1, and hyperphosphorylation of M1 leads to its retention in the nucleus (53). Also, the localization of M1 in the cell nucleus is time-specific (55). We coupled these two phenomena and found that the nuclear translocation of M1 was phosphorylation-dependent. Previously, several amino acids have been predicted to be phosphorylated by specific kinases such as proline kinase (Ser-53), protein kinase C (Ser-70 and Ser-161), and casein kinase (Ser-185) (Fig. 6A). The experiment with point mutants for all known phosphorylation sites of M1 (S53A, S70A, S161A, and S185A) along with the NLS deletion mutant revealed that unlike wild-type M1 protein, phosphorylation-specific mutants or NLS-M1 protein could not translocate to the nucleus (Fig. 6, B and C), suggesting that both NLS and phosphorylation is required for efficient nuclear translocation and survival gene activation (supplemental Fig. S5). This was further confirmed when the protein kinase C inhibitor calphostin C inhibited both phosphorylation and nuclear translocation of M1 protein (Fig. 6D). Confocal laser-scanning microscopy

**FIGURE 8.** Model for M1-mediated up-regulation of RelB-responsive survival genes (cIAP1, cIAP2, and cFLIP). A composite model is presented showing phosphorylation-mediated nuclear translocation of M1 followed by its binding with Daxx. Binding of M1 with Daxx results in disruption of Daxx-RelB-Dnmt interaction, resulting in binding of RelB to its promoter as well as promoter hypomethylation and transactivation of RelB-regulated genes (top and bottom right). In the absence of influenza virus infection (normal cell), Daxx interacts with the RelB bound to its target sites and recruits Dnmt3s, resulting in CpG hypermethylation (red spheres below DNA) of RelB target gene promoters, thus resulting in gene silencing (red cross) (bottom left). TSS, transcription start site.
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also revealed that when calphostin C was used during PR8 infection early nuclear accumulation of M1 was significantly reduced (Fig. 6F). Hence, it can be concluded that during early periods of influenza A infection M1 protein is phosphorylated by the cellular kinases and translocates from the cytoplasm to the nucleus where it binds with Daxx. The binding of Daxx by M1 abolishes RelB-Daxx complex formation, which prevents recruitment of Dnmts to RelB-responsive gene promoters, resulting in reduced CpG methylation of promoters and activation of survival genes (Fig. 8).

In addition to the role of M1 protein in modulating the repressor function of Daxx, up-regulation of Daxx protein was also observed in PR8-infected or M1-transfected cells (Fig. 7, A and B). Reorganization or degradation of Daxx by viruses has been reported previously (42–50). Although its expression has been shown to be induced by doxorubicin, UV-C irradiation, or hydrogen peroxide (64), PR8 infection had no effect on Daxx transcription (supplemental Fig. S1). The recruitment of Dnmts to RelB-responsive gene promoters, resulting in reduced CpG methylation of promoters and activation of survival genes (Fig. 8).

During early infection, it is crucial for virus to inhibit host-induced apoptotic stimuli for efficient infection. Thus, activation of the prosurvival genes by M1 protein may also help influenza A virus replicate better. To assess this, viral titers were measured following PR8 infection in the presence or absence of calphostin C. As expected, a significant reduction in viral titer was observed in PR8-infected cells treated with calphostin C compared with untreated PR8-infected cells (supplemental Fig. S6).

Thus, M1 protein has a dual function during the influenza A virus life cycle. During early infection, M1 protein activates prosurvival pathways to inhibit early apoptosis, but the same protein enhances the apoptotic machinery of cells during late infection (58). Although both functions of M1 are antagonistic in nature, they both help influenza A virus overcome the host defense for efficient viral propagation.

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