Identification of two residues in MCM5 critical for the assembly of MCM complexes and Stat1-mediated transcription activation in response to IFN-γ

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In response to IFN-γ, the latent cytoplasmic Stat1 (signal transducer and activator of transcription) proteins translocate into the nucleus and activate transcription. We showed previously that Stat1 recruits a group of nuclear proteins, among them MCM5 (minichromosome maintenance) and MCM3, for transcription activation. MCM5 directly interacts with the transcription activation domain (TAD) of Stat1 and enhances Stat1-mediated transcription activation. In this report, we identified two specific residues (R732, K734) in MCM5 that are required for the direct interaction between Stat1 and MCM5 both *in vitro* and *in vivo*. MCM5 containing mutations of R732/K734 did not enhance Stat1-mediated transcription activation in response to IFN-γ. In addition, it also failed to form complexes with other MCM proteins *in vivo*, suggesting that these two residues may be important for an interaction domain in MCM5. Furthermore, MCM5 bearing mutations in its ATPase and helicase domains did not enhance Stat1 activity. *In vitro* binding assays indicate that MCM3 does not interact directly with Stat1, suggesting that the presence of MCM3 in the group of Stat1TAD-interacting proteins is due to the association of MCM3 with MCM5. Finally, gel filtration analyses of nuclear extracts from INF-γ-treated cells demonstrate that there is a MCM5/3 subcomplex coeluting with Stat1. Together, these results strongly suggest that Stat1 recruits a MCM5/3 subcomplex through direct interaction with MCM5 in the process of IFN-γ-induced gene activation.

Most cytokines and growth factors use the JAK-STAT pathway for signaling (1–4). Upon ligand binding to cell surface receptors, the latent cytosolic signal transducer and activator of transcription (STAT) proteins are phosphorylated on a single tyrosine residue by the JAK tyrosine kinase, dimerize, and enter the nucleus, where they bind to specific DNA sequences and activate transcription. There are seven mammalian STAT genes, each performing a specific physiological role (3, 5). The STATs share common structural features, such as the DNA binding domain and SH2 domain, defined by crystal structures and molecular studies of these proteins (6–10). The very end of the carboxyl-terminal region of STATs is the transcription activation domain (TAD), although there is little sequence homology between them (11–15). Several STATs (Stat1, -3, -4, and -5) have a serine residue in their TADs, which has been shown to be phosphorylated in response to ligands and required for maximal transcription activity (16–18).

Interferons are involved in a variety of cellular functions such as antiviral responses, regulation of cell growth, and tumor surveillance (19–22). IFN-γ also plays an essential role in modulating the activities in adaptive immune responses (21, 22). Stat1 is the key transcription factor for the IFN-γ signaling pathway, demonstrated by the Stat1 knock-out mice, which were highly susceptible to microbial and viral pathogens and had a higher frequency of tumor occurrence (23–25).

Upon IFN-γ stimulation, Stat1 proteins become phosphorylated on tyrosine 701, homodimerize, enter the nucleus, and bind to a DNA sequence called GAS (gamma interferon activated sites) (26–28). There are two naturally occurring forms of Stat1, α and β, due to alternative splicing (29). The β form lacks the 38-residue TAD of Stat1α and is transcriptionally inactive, even though it can be phosphorylated on tyrosine, form dimers, enter the nucleus, and bind DNA (29–31). In this 38-residue TAD, there is a short sequence motif LPMSP that is also present in Stat3, -4, and -5 (16). In addition to the tyrosine phosphorylation, the serine residue at position 727 in the LPM*S*P motif is also phosphorylated in response to IFN-γ and is required for the maximal transcription activity of Stat1α (16). When serine-727 is mutated to alanine, IFN-γ-induced transcription, which utilizes Stat1α homodimer, is reduced to $\approx$20% of the wild-type level (14, 16).

We have previously reported that the Stat1TAD interacts with a specific group of nuclear proteins, among them CBP/p300 (32, 33), MCM5, and MCM3 (14). MCM5 and MCM3 are two members of the minichromosome maintenance (MCM) family required for DNA replication (34–38). The MCM proteins form complexes of various constitutions and have conserved helicase domains (39). The interaction between Stat1 and MCM5 is direct, depends on Ser-727 of Stat1, and is enhanced by phosphorylation of Ser-727 (14).

In this report, we identified the critical residues in MCM5 that mediate its interaction with Stat1. Specifically, a double mutation of R732 and K734 in the C terminus of MCM5 results in a dramatic decrease in its affinity for binding to Stat1 both *in vitro* and *in vivo*. In contrast to wild-type MCM5 proteins that enhance Stat1 activity, MCM5 R732/K734 mutant proteins fail to enhance Stat1-mediated transcription activation. MCM5 bearing mutations in its ATPase and helicase domains also failed to enhance Stat1 activity. In addition, *in vitro* binding assays demonstrate that Stat1 does not interact with MCM3 directly, suggesting that the presence of MCM3 in the group of Stat1TAD-interacting proteins is due to the association of MCM3 with MCM5. Coimmunoprecipitation experiments showed that the MCM5 R732/K734 mutant does not bind Stat1 or other MCM proteins *in vivo*, suggesting that these two residues may be important for a critical interaction domain in MCM5. Furthermore, gel filtration analyses of nuclear extracts from INF-γ-treated cells demonstrate that there is a distinct subcomplex of MCM5/3 in the nucleus and Stat1 coelutes with this MCM5/3 subcomplex. All together, these results strongly sug-

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Abbreviations: Stat1, signal transducer and activator of transcription; MCM, minichromosome maintenance; TAD, transcription activation domain; GST, glutathione *S*-transferase; HA, hemagglutinin A.

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gest that Stat1 recruits a MCM5/3 subcomplex through direct interaction with MCM5 for transcription activation in response to IFN-γ.

Materials and Methods

Cell Culture and Antibodies. 2fTGH and U3A cells (provided by G. Stark, Cleveland Clinic Foundation Research Institute, Cleveland, OH, and I. Kerr, Imperial Cancer Research Foundation, London, United Kingdom) were maintained in DMEM supplemented with 10% cosmic calf serum. U-2 OS human osteosarcoma cells and BUD-8 human primary fibroblasts (American Type Culture Collection) were maintained in DMEM supplemented with 10% FBS. All types of serum were from HyClone. 2fTGH stable cell lines containing various hemagglutinin A (HA)-tagged MCM5 plasmids were maintained in media with 1 mg/ml G418. The human MCM5-specific polyclonal antibody was made against bacterially expressed full-length MCM5 proteins (Covance Research Products). Antibodies against MCM2, -3, -4, -6, or -7 were kindly provided by R. Knippers (University of Konstanz, Konstanz, Germany). For immunoprecipitation, an antibody against the amino terminus of Stat1 was purchased from Transduction Laboratories (Lexington, KY). For Western blotting, a Stat1 antibody was purchased from Research Diagnostics. Anti-HA antibody was from Roche Molecular Biochemicals. Recombinant human IFN-γ was a gift from Amgen Biologicals. Cells were treated with IFN-γ at 5 ng/ml for lengths of time as indicated in each experiment. G418 sulfate was from Calbiochem.

Glutathione *S*-transferase (GST) Pull-Down Assays, Coimmunoprecipitation, and Western Blot Analysis. GST fusion proteins were purified from bacteria with glutathione-Sepharose beads (Amersham Pharmacia). *In vitro* translation reactions were done by using the TNT T7 system (Promega). Preparation of nuclear extracts and GST pull-down assays were done as previously described (14). For coimmunoprecipitation experiments, 0.5 mg of nuclear extracts from IFN-γ-treated (30 min) cells were incubated overnight with 25 μg of anti-HA antibodies in buffer BC100 (14). Immune complexes were brought down with protein A/G agarose beads (Santa Cruz Biotechnology), washed with the same buffer, and separated by SDS/PAGE. Western blot analyses were done with chemiluminescence (DuPont/NEN).

Plasmid Constructions. GST-Stat1TAD was constructed as previously described (14). Bacteria expression vector pRSETB (Invitrogen) containing wild-type MCM5 or MCM3 cDNA was provided by R. Knippers (University of Konstanz) and used for *in vitro* translation. The cDNA of MCM5 was further subcloned into pBluescript (Stratagene) to generate SK/MCM5. Point mutations in MCM5 were generated with oligonucleotides containing the appropriate mutations, with the use of the Gene-Editor kit from Promega. The HA epitope was placed at the N terminus of MCM5 by PCR with the following overlapping oligonucleotides for the 5′ primer: 5′AAGGAAAAAAGCGGCCGCACCATGGCATATCCATACGATGTGCCAGACTACGCG, 5′TACGATGTGCCAGACTACGCGTCGGGATTCGACGATCCTGG, and 5′TTCAGGCTACGAATGCTGGAAGGGCTG for the 3′ primer to generate SK/HAMCM5. The HA-tagged MCM5 cDNA was then subcloned into the RcCMV expression vector (Invitrogen). GST/Stat1β was provided by J. Darnell (Rockefeller University, New York). GST/Stat1α was generated by subcloning the Stat1TAD fragment into GST/Stat1β. The 3xLy6E luciferase reporter was constructed as previously described (16).

Transfection Experiments. Transient transfections of U-2 OS cells were done as previously described (14). Luciferase assays were performed with the Dual-Luciferase Reporter System with the TK Renilla luciferase reporter as an internal control (Promega).

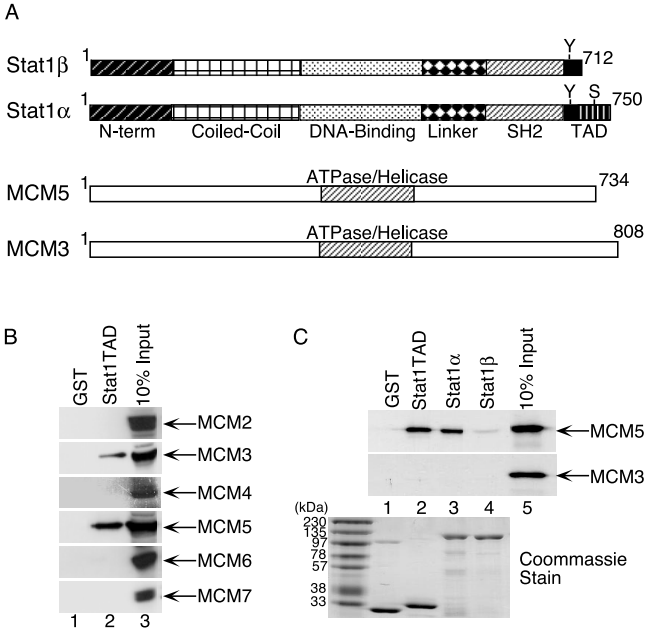

Fig. 1. GST-Stat1TAD interacts directly with MCM5, but not MCM3. (*A*) Schematic view of Stat1α and -β and MCM5 and MCM3. The domain structure of Stat1 is according to Chen *et al.* (7). TAD, transcription activation domain; Y, Tyr-701; S, Ser-727. (*B*) MCM5 and MCM3 are among the group of Stat1TAD-interacting nuclear proteins. Nuclear extracts from U3A cells were incubated with Sepharose-bead-bound GST or GST-Stat1TAD fusion proteins. The bound proteins were separated by SDS/PAGE and analyzed by Western blotting with indicated antibodies. (*C*) Stat1TAD interacts directly with MCM5, but not with MCM3. ^{35}S-labeled MCM5 or MCM3 proteins were translated *in vitro* and incubated with the indicated GST fusion proteins bound to Sepharose beads. The bound proteins were separated by SDS/PAGE and visualized by autoradiography. The bottom panel shows the various GST fusion proteins separated by SDS/PAGE and visualized by Coomassie staining.

All results shown are luciferase activities normalized against the internal control Renilla luciferase reporter (Promega). 2fTGH stable cell lines containing various mutant MCM5 proteins were generated with the calcium-phosphate transfection kit (GIBCO/BRL and Life Technologies, Grand Island, NY). Twenty-four hours after transfection, cells were incubated in selection media containing 1 mg/ml G418, and positive clones were identified by Western blotting with αHA antibodies.

Gel Filtration Chromatography. Nuclear extract (1.5 ml) was dialyzed into BC100 (14) with 10% glycerol, then concentrated down to 300 μl with a Millipore Ultrafree-0.5 centrifugal filter. The concentrated sample (200 μl) was applied at a flow rate of 0.2 ml/min to a Superose 6 HR10/30 column preequilibrated in the same buffer at 4°C with the AKTA FPLC system (Amersham Pharmacia). Proteins were eluted in the same buffer, and 1.0-ml fractions were collected and then lyophilized to a final volume of 250 μl. A molecular weight calibration curve for the Superose 6 HR10/30 column was generated with the HMW gel filtration calibration kit (Amersham Pharmacia) according to the instructions of the manufacturer.

Results

Our previous analyses of Stat1TAD-interacting proteins revealed that Stat1TAD interacted directly with MCM5 (14) (schematic views of Stat1, MCM5, and MCM3 are shown in Fig. 1*A*). To further determine whether other members of the MCM family can interact with Stat1TAD, nuclear proteins bound to GST or GST-Stat1TAD fusion proteins were separated by SDS/PAGE and analyzed by Western blotting (Fig. 1*B*). Only MCM5 and MCM3

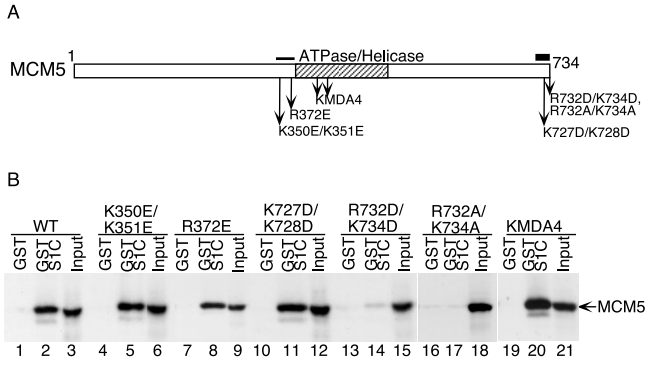

Fig. 2. MCM5 protein containing mutations of R732 and K734 cannot interact with Stat1TAD. (*A*) Point mutations in MCM5./indicates a double mutation of residues; KMDA4 is a quintuplet mutation of the ATP-binding site K387 into Met and the conserved DEFD motif (445–449) into four Ala. Lines on top of the schematic MCM5 molecule indicate previously reported regions of MCM5 that were required for binding to Stat1TAD, with the thickness of the lines representing the strength of interaction. (*B*) Wild-type or mutant MCM5 proteins were labeled with ^{35}S by *in vitro* translation and incubated with Sepharose-bead-bound GST or GST-Stat1TAD fusion proteins (GSTS1C). The proteins bound to beads were separated by SDS/PAGE and visualized by autoradiography. Input lanes contain 10% of total input.

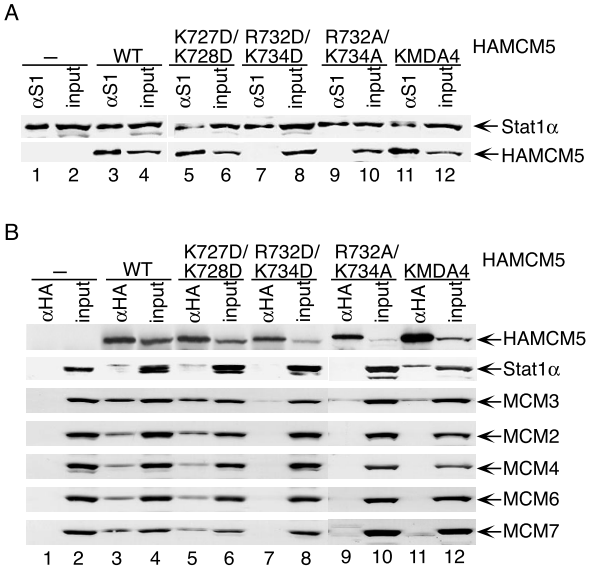

Fig. 3. The R732/K734 mutant MCM5 does not interact with Stat1 and other MCM proteins *in vivo*. (*A*) Wild-type or mutant MCM5 was tagged at the N terminus with the HA epitope and transiently transfected into 2fTGH cells. Stat1 proteins from whole-cell extracts were precipitated with a Stat1 antibody, and the immune precipitates were separated by SDS/PAGE and analyzed by Western blotting with the indicated antibodies. Input lanes contain 10% of total input. (*B*) Nuclear extracts from stable cell lines containing the various HA-tagged MCM5 proteins were prepared from cells treated with IFN-γ for 30 min. The HA-tagged MCM5 proteins were precipitated with the anti-HA antibody, and the immune precipitates were separated by SDS/PAGE and analyzed by Western blotting with the indicated antibodies. Input lanes contain 20% of total input.

were present in the group of Stat1TAD-interacting nuclear proteins (Fig. 1*B*, Lane 2). To determine whether the TAD was the only region in Stat1 that interacted with MCM5 and whether MCM3 directly interacted with Stat1, GST fusion proteins containing Stat1α, Stat1β, and Stat1TAD were used in a GST pull-down assay with *in vitro* translated and ^{35}S-labeled MCM5 or MCM3. Stat1TAD interacted with MCM5 (Fig. 1*C*, *Top*, Lane 2), whereas GST alone did not (Fig. 1*C*, *Top*, Lane 1). Full-length Stat1α interacted with MCM5 well, whereas Stat1β did not (Fig. 1*C*, *Top*, Lanes 3 and 4), indicating that the Stat1TAD is the only region in Stat1 that interacted with MCM5. In contrast, MCM3 did not interact with any form of Stat1 directly (Fig. 1*C*, *Middle*). Equal amounts of GST fusion proteins were used (Fig. 1*C*, *Bottom*). Earlier studies have shown that MCM5 and MCM3 preferentially interact with each other and form a subcomplex *in vivo* (40, 41). Together, these results suggest that the presence of MCM3 in the group of Stat1TAD-interacting proteins is due to the association of MCM3 with MCM5 and that the MCM5/3 subcomplex is recruited by Stat1TAD through direct interaction with MCM5.

Previous deletion analysis of MCM5 indicated that residues 350–400 had a low level of Stat1-binding affinity, and the C terminus of MCM5 is required for maximal Stat1 binding (14). Because phosphorylation of Stat1 Ser-727 enhanced interaction with MCM5, we hypothesized that phospho-Ser-727 interacted with positively charged residues in MCM5. Site-directed mutagenesis was used to mutate seven positively charged residues in the Stat1-binding region of MCM5, specifically K350, K351, R372, K727, K728, R732, and K734. These residues were mutated to negatively charged residues (D or E), either individually or two at a time if they were next to each other (Fig. 2*A*). Residues R732 and K734 were also mutated to Ala. Mutations (KMDA4, Fig. 2*A*) were also generated in the ATP-binding site (K387M) and the conserved DEFD domain (445–449 to Ala), mutations of which have been shown to reduce the ATPase/helicase activity for the MCM4/6/7 subcomplex (42). MCM5 proteins containing these point mutations were generated by *in vitro* translation and assayed for their ability to bind to Stat1TAD in a GST pull-down assay (Fig. 2*B*). Of all the mutant MCM5 proteins, only two that contained mutations of R732/K734, either to Asp or to Ala, lost their ability to interact with Stat1TAD (Fig. 2*B*, Lanes 14 and 17), indicating that these two residues are important for interaction between MCM5 and Stat1TAD.

To demonstrate the interaction between MCM5 and full-length Stat1α *in vivo*, wild-type or mutant MCM5 proteins tagged with the hemagglutinin A (HA) epitope at the N terminus were transiently expressed in 2fTGH cells. Endogenous Stat1 proteins were precipitated by a Stat1 antibody, and the immunoprecipitated complexes were analyzed by Western blotting with the anti-HA antibody (Fig. 3*A*). Consistent with the *in vitro* binding data (Fig. 2*B*), the wild-type MCM5 as well as the K727D/K728D and KMDA4 mutant MCM5 (which also serve as positive controls) could interact with Stat1 well (Fig. 3*A*, Lanes 3, 5, and 11). However, the two mutants of R732/K734 failed to interact with Stat1 *in vivo* (Fig. 3*A*, Lanes 7 and 9). To further study the *in vivo* interactions between MCM5, Stat1, and other MCM proteins, stable cell lines were generated that contained the HA-tagged wild-type or mutant MCM5. Nuclear extracts from IFN-γ-treated cells were immunoprecipitated with anti-HA antibodies, and the immunoprecipitated complexes were separated by SDS/PAGE followed by Western blotting. Similar amounts of HA-tagged MCM5 proteins were precipitated from all of the cell lines (Fig. 3*B*, Lanes 3, 5, 7, 9, and 11). Because of the competition from endogenous MCM5 proteins, the amount of Stat1 proteins bound to HA-tagged MCM5 proteins is less than previously reported for coimmunoprecipitation experiments (14). Wild-type HAMCM5 and HAMCM5 containing K727D/K728D coimmunoprecipitated Stat1 and other MCM proteins well (Fig. 3*B*, Lanes 3 and 5). HAMCM5 containing R732D/K734D or R732A/K734A did not coimmunoprecipitate Stat1 (Fig. 3*B*, Lanes 7 and 9). The R732D/K734D or R732A/K734A mutant MCM5 also failed to interact with all of the other MCM proteins (Fig. 3*B*, Lanes 7 and 9). Interestingly, although the KMDA4 mutant could coimmunoprecipitate

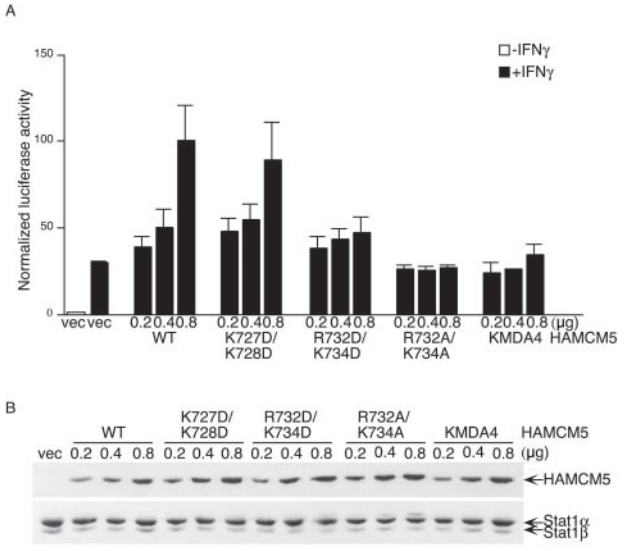

Fig. 4. Specific interaction between Stat1 and MCM5 is required for MCM5 to enhance Stat1-mediated transcription activation. (*A*) Expression plasmids containing HA-tagged wild-type or mutant MCM5 were transiently transfected into U-2 OS cells together with a Stat1-dependent luciferase reporter and the internal control Renilla luciferase reporter (dual luciferase reporter system; Promega). Twenty-four hours after transfection, the cells were either left untreated or treated with IFN-γ for 6 h and harvested for luciferase assays. Results shown are luciferase activities normalized against internal control and the mean + SD of three to five experiments. For MCM5 overexpression samples, only results from treated cells are shown. Vec, the RcCMV plasmid. (*B*) Western blotting analyses were done with 10 μl of cell lysates used for luciferase assays above. Only lysates from treated cells are shown.

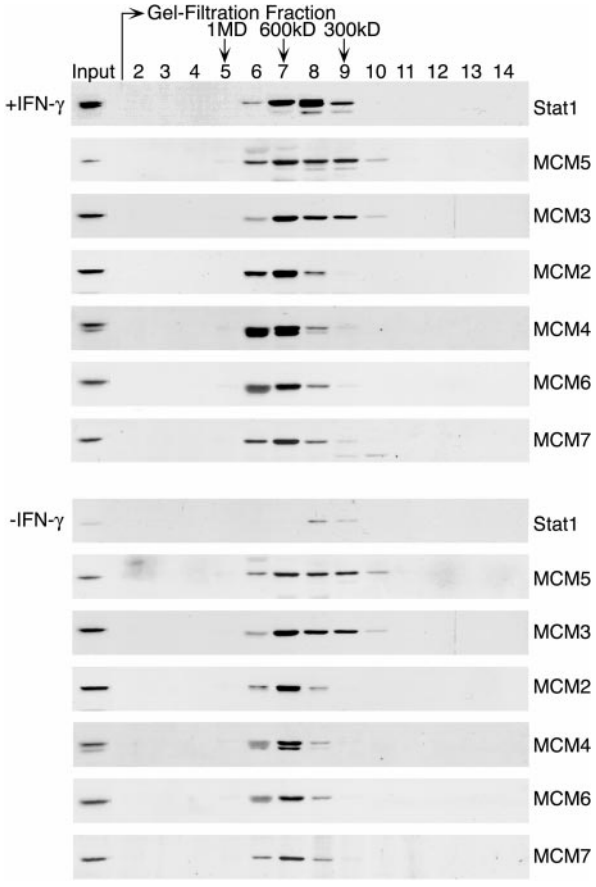

Fig. 5. Coelution of Stat1 and the MCM5/3 subcomplex. Nuclear extracts from IFN-γ-treated (30 min) BUD-8 cells were fractionated by FPLC on a Superose 6 HR10/30 column. 2.5% of total input and 25% of each fraction (fraction numbers are at the top of each lane) were analyzed by Western blotting with the indicated antibodies. Molecular masses were calculated from a calibration curve generated with the Pharmacia HMW calibration kit.

Stat1, it interacted with MCM3 less well and did not bind other MCMs (Fig. 3*B*, Lane 11). These results suggest that residues R732 and K734 may be important for a critical interaction domain in MCM5.

We previously reported that an increase in the level of nuclear MCM5 proteins correlates with an enhancement of Stat1-mediated transcription activation in response to IFN-γ (14). To see whether the mutant MCM5 defective in binding to Stat1 can enhance transcription, the HA-tagged wild type or mutant MCM5 proteins were overexpressed transiently in U-2 OS cells together with the IFN-γ-inducible luciferase reporter containing three copies of Stat1-binding site 3xLy6E (14, 16). Twenty-four hours after transfection, the cells were treated with IFN-γ for 6 h or left untreated before luciferase assay. IFN-γ treatment induced a reporter activity about 30-fold higher than that of untreated cells (Fig. 4*A*). Overexpression of wild-type MCM5 protein and the K727D/K728D mutant, which could still bind to Stat1, enhanced the IFN-γ-induced reporter activity in a dose-dependent manner, whereas the R732D/K734D or R732A/K734A mutants, which are defective in binding to Stat1, did not enhance that reporter activity (Fig. 4*A*). The KMDA4 mutant that could still bind to Stat1, but not to other MCMs, did not enhance Stat1 activity either (Fig. 4*A*). Overexpression of MCM5 did not affect the baseline reporter activity in untreated cells (data not shown). The various types of HA-tagged MCM5 proteins were overexpressed at similar levels in the transfected cells (Fig. 4*B*, *Upper*). The levels of endogenous Stat1 were similar in all of the transfected cells (Fig. 4*B*, *Lower*). IFN-γ treatment did not affect the level of MCM5 expression (data not shown). Furthermore, overexpression of MCM3 alone or together with MCM5 did not further enhance Stat1 activity (data not shown). Taken together, these results suggest that direct interaction between Stat1 and MCM5 is required for MCM5 to enhance Stat1-mediated transcription activation in response to IFN-γ.

Earlier studies using immunoprecipitation, gradient centrifugation, and gel filtration techniques have shown that the MCM proteins form various subcomplexes composed of different members of the family, such as MCM5/3 or MCM4/6/7 subcomplexes at varying salt concentrations (40, 41, 43–46). To see the pattern of MCM complexes and Stat1 in IFN-γ-treated cells, nuclear extracts were prepared from BUD-8 cells untreated or treated with IFN-γ for 30 min and subjected to gel filtration followed by SDS/PAGE and Western blotting analyses. The nuclear extracts were prepared with buffers containing 320 mM KCl and further dialyzed into buffers containing 100 mM KCl for gel filtration. Stat1 eluted in fractions between 300 kDa and 600 kDa (Fig. 5, *Upper*, Fractions 7–9), which is larger than the expected size of a dimer of 180 kDa (9), suggesting that Stat1 proteins are present in a complex with other nuclear proteins *in vivo*. MCM5 and MCM3 proteins eluted in a pattern with a broad peak between 300 kDa and 600 kDa (Fig. 5, Fractions 7–9), similar to that of Stat1. In striking contrast to MCM3 and MCM5, MCM2, -4, -6, and -7 eluted with a narrow peak at 600 kDa (Fig. 5, Fractions 6 and 7). These results indicate that in addition to being part of the hexamer complex (≈600 kDa) with the other MCM proteins, MCM5 and MCM3 also exist as a distinct subcomplex of ≈450 kDa (Fig. 5, Fractions 8 and 9), even in low-salt concentrations. In untreated BUD-8 cells, very little Stat1 is in the nucleus (Fig. 5, *Lower*), and none of the patterns

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of the MCM proteins were qualitatively different from those of the IFN-γ-treated cells (Fig. 5, *Upper*). However, in IFN-γ-treated cells, there was some increase in the level of MCM2, -4, -6, and -7 in the fraction of >600 kDa (Fig. 5, *Upper*, Fraction 6). This may be caused by a small number of Stat1 interacting with the MCM5 molecules in the hexamer, resulting in the increase in the molecular mass of the complex, because a low level of Stat1 was detected in fraction 6 in IFN-γ-treated cells (Fig. 5, *Upper*, Fraction 6). All together, these results indicate that in the nucleus of IFN-γ-treated cells, Stat1 is present in a complex with other proteins, and the elution patterns of Stat1 and MCM5/3 is consistent with data suggesting that Stat1 recruits a MCM5/3 subcomplex for gene activation in response to IFN-γ.

Discussion

The MCM family is a group of proteins conserved through all eukaryotes and archaebacteria (47) and required for DNA replication (34–37). The MCMs form complexes of various constitution (35, 40, 44), assemble at the initiation site of DNA replication along with other protein complexes such as origin–recognition complex (48), and travel together with the replisome (49, 50). The MCMs have common structural features such as DNA-dependent ATPase domains characteristic of DNA helicases (39). They have been shown to change the structure of chromatin (44, 51) and unwind DNA with their potential helicase activity (42, 52). Our earlier studies suggested that MCM5 is involved in transcription activation through a direct interaction with a transcription factor, Stat1 (14). Recently it was also shown that the family of MCM proteins is associated with RNA II polymerase holoenzyme (53), further suggesting that these MCM proteins are not only required for DNA replication but are also involved in transcription. It has been shown that the numbers of MCM proteins are 50–500 times higher than the number of replication origins in yeast (43, 54). It is conceivable that the helicase activities of these abundant MCM proteins are used by at least two different biological processes that require the unwinding of the DNA duplex, i.e., DNA replication and RNA transcription. The MCM proteins can be directed to a specific process through interactions with other proteins such as histones (51) and origin–recognition complex 1 (49, 55, 56) for DNA replication, or the C-terminal domain of RNA polymerase II (53) and the transcription activation domain of Stat1 for transcription activation.

It is well established that, in addition to the hexamer complex containing one of each of the six MCMs (40, 46), there are many different MCM subcomplexes containing different members of the family, such as MCM5/3 (40, 41), MCM4/6/7 (42, 46), and MCM2/4/6/7 (45, 46, 51). Although the hexamer is considered to be the complex required for DNA replication (46), it is not clear whether the subcomplexes have specific functions or are simply the intermediates for the complete assembly of the hexamer complex (46). The separation of MCM proteins into subcomplexes occurs all through evolution, suggesting a potential functional importance for these subcomplexes. A multistep assembly process can provide many check points for regulation of DNA replication, and/or the different subcomplexes can be used by other pathways. In this report, we demonstrated a specific recruitment of the MCM5/3 subcomplex by Stat1 for IFN-γ-induced transcription, mediated by Ser-727, Leu-724 of Stat1 (14) and Arg-732, Lys-734 of MCM5. Together with our previous studies showing that phosphorylation of Ser-727 in Stat1 enhances binding to MCM5, these results indicate an association between Stat1 and MCM5, possibly mediated by an electrostatic interaction. These results also provide one explanation for the requirement of Ser-727 for the maximal Stat1 activity, i.e., phosphorylation of Ser-727 enhances interaction between Stat1 and other nuclear proteins, such as MCM5, for transcription activation.

The involvement of MCM5/3 in Stat1-mediated transcription activation is further supported by the results showing that this enhancement of Stat1 activity by MCM5 depends on the direct interaction between MCM5 and Stat1. Mutant MCM5 proteins defective in binding to Stat1 are unable to enhance Stat1 activity. Furthermore, MCM5 proteins with mutations in the ATPase and helicase domains cannot enhance Stat1 activity either. Because this ATPase/helicase mutant does not bind to other MCMs well, it is not clear whether the ATPase/helicase function of MCM5 *per se* is required for enhancement of Stat1 activity. An *in vitro* transcription system for Stat1 with chromatin template needs to be developed for further structural and functional analyses.

Although it is clear that MCM5/3 is involved in transcription, it also remains a distinct possibility that Stat1 or Stat1-mediated transcription activity may have a direct effect on DNA replication. It has been reported that the retinoblastoma protein inhibited DNA replication through association with MCM7 (57). It is well known that IFN-γ inhibits cell growth by slowing down the transition from G_1 to S phase and that this inhibition requires a transcriptionally active Stat1, e.g., Stat1 containing S727A mutation, which cannot bind to MCM5 (14), loses its ability to inhibit cell growth (58). It is possible that by recruiting the MCM5/3 subcomplex to the process of transcription activation, Stat1 is directly interfering with DNA replication through competition for MCM5/3 or inhibiting the assembly of the MCM hexamer. These possibilities remain to be explored by gel filtration analyses of purified MCM proteins and *in vitro* DNA replication systems.

Finally, the R732/K734 mutant MCM5 failed to interact with any of the other MCM proteins *in vivo*, suggesting that these two residues may be important for forming a protein–protein interaction surface in MCM5. It is conceivable that MCM5 only contacts one or two of the other MCM proteins, such as MCM3, in the hexamer complex, and the loss of binding to MCM3 could result in the complete dissociation of MCM5 from the MCM hexamer. Residue R732 is conserved from yeast to human, and K734 is conserved in *Xenopus*, mouse, and human. It has been suggested that the C-terminal region of MCM proteins may have a conserved helical structure (37, 59). Search of the PROSITE database (60) resulted in a predicted protein structure of MCM5 with the R732 and K734 residues in a short β-sheet following several long α-helices in the C terminus of MCM5. Perhaps the short β-sheet is important for the formation of an interaction domain composed of several α-helices that contacts Stat1 and other MCM proteins. Domains containing α-helices have been shown to be protein–protein interaction surfaces for contacting other proteins. For example, the basic leucine zipper proteins forms dimers from various monomers through interactions between α-helices in the basic leucine zipper domain, and the formation of different heterodimers of these basic leucine zipper transcription factors results in the binding to similar but distinct DNA target sequences (61). In the case of MCM5 and Stat1, the regulation of protein–protein interactions between MCMs and Stat1 can contribute to two diverse and important cellular processes, DNA replication and cytokine-induced transcription.

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