Inhibition of Cell Proliferation by the Mad1 Transcriptional Repressor

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Mad1 is a basic helix-loop-helix-leucine zipper protein that is induced upon differentiation of a number of distinct cell types. Mad1 dimerizes with Max and recognizes the same DNA sequences as do Myc:Max dimers. However, Mad1 and Myc appear to have opposing functions. Myc:Max heterodimers activate transcription while Mad:Max heterodimers repress transcription from the same promoter. In addition Mad1 has been shown to block the oncogenic activity of Myc. Here we show that ectopic expression of Mad1 inhibits the proliferative response of 3T3 cells to signaling through the colony-stimulating factor-1 (CSF-1) receptor. The ability of over-expressed Myc and cyclin D1 to complement the mutant CSF-1 receptor Y809F (containing a Y-to-F mutation at position 809) is also inhibited by Mad1. Cell cycle analysis of proliferating 3T3 cells transfected with Mad1 demonstrates a significant decrease in the fraction of cells in the S and G₂/M phases and a concomitant increase in the fraction of G₁ phase cells, indicating that Mad1 negatively influences cell cycle progression from the G₁ to the S phase. Mutations in Mad1 which inhibit its activity as a transcription repressor also result in loss of Mad1 cell cycle inhibitory activity. Thus, the ability of Mad1 to inhibit cell cycle progression is tightly coupled to its function as a transcriptional repressor.

The basic helix-loop-helix-leucine zipper-containing transcription factors Mad1 and c-Myc each form sequence-specific DNA-binding heterodimers with the Max protein but appear to have opposing transcriptional functions (1, 3, 6, 25). Mad1 is a transcriptional repressor which is expressed at low levels in proliferating cells and induced during differentiation of several distinct cell lineages in vitro and in vivo (e.g., hematopoietic, neuronal, and epithelial cells) (2, 10, 18, 19, 22, 39). By contrast, the expression of the *c-myc* proto-oncogene is primarily restricted to proliferating cells and is downregulated during differentiation (1, 6, 25). Recent studies indicate that transcriptional repression by Mad1 is mediated through interaction with another protein in addition to Max. All four Mad family proteins possess a conserved N-terminal domain that mediates association with mSin3A and mSin3B, the mammalian counterparts of the yeast corepressor Sin3. mSin3, Mad1, and Max form a ternary complex on DNA, and mutations in Mad1 that disrupt its interaction with either Max, mSin3, or DNA result in the failure to block c-Myc cotransformation and the loss of transcriptional repression (4, 19, 20, 35).

The inverse regulation of c-Myc and Mad1 as well as their opposing transcriptional properties suggests that Mad1 may antagonize the function of Myc in controlling proliferation and differentiation. Support for this hypothesis derives from the demonstration that expression of Mad1 and other Mad family proteins (Mxi1, Mad3, and Mad4) inhibits cotransformation of primary cells by Myc and Ras (7, 19–21, 39). Moreover, loss of heterozygosity of Mxi1 occurs in some prostate cancers, sug-

gesting that Mxi1 and possibly other Mad family proteins act as tumor suppressors (11). However, it is unclear what role Mad expression plays during differentiation and how Mad1 exerts its inhibitory effects on the growth of transformed cells. To begin to address these questions we have examined the effects of ectopic Mad1 expression on proliferating cells and have focused on the response of quiescent cells to signaling through the colony-stimulating factor-1 (CSF-1) receptor.

Previous work has shown that quiescent 3T3 cells expressing the human CSF-1 receptor (CSF-1R) can be stimulated to proliferate upon treatment with CSF-1 ligand (32, 33). As expected, these cells induce immediate-early response genes, including *jun*, *fos*, and *c-myc*, during the G₀-to-G₁ transition. However, CSF-1 treatment of 3T3 cells bearing a mutant CSF-1R (CSF-1R Y809F, containing a Y-to-F mutation at position 809) leads to only weak induction of *c-myc* and loss of the proliferative response, even though normal levels of *jun* and *fos* mRNAs are expressed. Importantly, the proliferative response to CSF-1 can be restored following transfection with a *c-myc* expression vector, suggesting that c-Myc is necessary, but not sufficient, for these cells to enter the cell cycle (32). More recent studies indicate that c-Myc and cyclin D1 collaborate during the mitogenic response (34). Cyclin D1 mRNA, which is also induced upon CSF-1 stimulation of cells bearing wild-type CSF-1R, is not expressed in CSF-1R Y809F cells following CSF-1 treatment. However, ectopic *c-myc* expression, in addition to reconstituting the proliferative response to CSF-1, facilitates cyclin D1 induction (34). As with c-Myc, enforced expression of cyclin D1 can complement mitogenic signaling by the mutant receptor, and c-Myc is reinduced in cyclin D1-rescued cells, albeit with slower-than-normal kinetics and to lower levels. The CSF-1-stimulated entry of cyclin D1-rescued cells into the division cycle requires c-Myc and vice versa (34). Thus, both of the genes that encode these proteins are required for progression from G₀ into S phase, cross-

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FIG. 1. Effect of Mad1 overexpression on CSF-1 mitogenesis. (A) 3T3 cell lines stably expressing human CSF-1R were infected with retroviruses encoding the Mad1 protein listed at the bottom of the panel or with a retrovirus lacking a cDNA insert (Mock). The percentage of cells capable of forming colonies in soft agar relative to the number of cells plated is reported. The error bars represent the standard deviation of the mean from at least three independent experiments. (B) The [³⁵S]methionine-labeled Mad1 proteins from cells infected with the indicated retrovirus and then subjected to immunoprecipitation and analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis are shown. Sizes of the molecular mass markers are shown on the left.

regulate each other's expression, and do not function in a strictly linear signaling pathway.

Because the response of CSF-1R-expressing 3T3 cells to CSF-1 has been reasonably well characterized and shown to be dependent upon *c-myc*, they provide a good system to initiate studies on the effects of ectopic *mad1* expression on cell cycle progression. In a recent paper, published while this work was in progress, it was shown that ectopic expression of Mad can slow the growth of certain tumor cell lines (9). We show here that Mad1 blocks the cell cycle progression of untransformed cells responding to a mitogenic stimulus, and that mutations in Mad1 which cancel its activity as a transcriptional repressor result in loss of its cell cycle-inhibitory function.

MATERIALS AND METHODS

Construction of Mad1 expression vectors and retrovirus production. *Eco*RI fragments containing the complete Mad1 cDNA coding sequence or their mutant derivatives Mad1 Δ LZ and Mad1 L12P/A16P were subcloned into the *Eco*RI site of the bicistronic pSR α -MSV-TKneo retrovirus vector (26) or into the *Cla*I site of pSR α -MSV-TKCD8 (17) by blunt-end insertion. In both vectors, Mad1 expression was driven by the viral long terminal repeat promoter of Moloney murine leukemia virus, whereas the neomycin resistance gene (*neo*) and the gene encoding the mouse T-cell coreceptor CD8 were under control of the thymidine kinase promoter. For virus production, vectors coexpressing Mad1 and *neo* were cotransfected (8, 28) together with a plasmid encoding an ecotropic helper virus containing a defective virion-packaging ψ 2 sequence (26). Culture supernatants containing retroviruses were harvested 48 to 72 h after transfection and were used to infect proliferating NIH 3T3 mouse fibroblasts expressing either wild-type CSF-1R or mutant CSF-1R Y809F alone or CSF-1R Y809F plus Myc or cyclin D1 (34). Cells were plated 72 h after infection onto soft agar medium containing 15% serum and purified human recombinant CSF-1 at 2,000 U/ml (1 U = 0.44 fmol). Colonies were counted 14 and 21 days after plating. No colonies appeared in the absence of CSF-1; cells expressing the CSF-1R Y809F mutant do not form colonies in the presence of the growth factor unless rescued by Myc or cyclin D1 (34).

Cell labeling. Forty-eight hours following infection with Mad1 retroviruses, 10⁶ cells were metabolically labeled for 1 h with 0.2 mCi of [³⁵S]methionine (800 Ci/mmol; Amersham) per ml in 1 ml of methionine-free medium supplemented with dialyzed 10% fetal calf serum. Lysates were prepared and immunoprecipitations were performed as described previously (2).

Cell cycle analysis. Vectors coexpressing either wild-type or mutant Mad1 and CD8 were transfected into NIH 3T3 cells (8), and 48 h later, transfected cells were centrifuged and resuspended in 50 μ l of a titrated excess of either mouse CD8-specific antibody LY2 (Caltag, South San Francisco, Calif.) or an isotype-matched control rat immunoglobulin G2b and incubated for 30 min on ice. After being washed twice with cold phosphate-buffered saline (PBS), cells were resus-

pended in 50 μ l of a titrated excess of fluorescein-conjugated goat anti-rat immunoglobulin (BioSource International, Camarillo, Calif.) and incubated for 30 min on ice. After one wash in cold PBS, cells were resuspended in 200 μ l of PBS, and 600 μ l of ice-cold 95% ethanol was added dropwise while the sample was being vortexed. After a 20-min incubation on ice, cells were centrifuged and resuspended in 1 ml of PBS containing 0.05 mg of propidium iodide per ml. Immediately prior to analysis by flow cytometry, each sample was treated for 30 min at room temperature with RNase (Calbiochem, San Diego, Calif.) at a final concentration of 50 μ g/ml and filtered through a 44- μ m-pore-size mesh. Approximately 75,000 cells from each sample were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.), collecting forward- and side-scatter green fluorescence from fluorescein isothiocyanate-labeled CD8 molecules and red fluorescence from propidium iodide-stained DNA. DNA content histograms of cells expressing transfected genes were obtained by using electronic gating to select those cells with CD8-fluorescein isothiocyanate fluorescence greater than the background level (17, 23). The percentages of cells within the G₁, S, and G₂/M phases of the cell cycle were determined by analysis of the single-parameter DNA histogram with the computer program ModFit (Verity Software House, Topsham, Maine).

RESULTS

Mad1 expression blocks CSF-1-induced mitogenesis of NIH 3T3 cells. Introduction of the human CSF-1R into mouse NIH 3T3 fibroblasts enables them to form colonies in semisolid medium containing human recombinant CSF-1 (33). Typically, 10 to 15% of CSF-1R cells form colonies in a manner that is absolutely dependent on the presence of CSF-1 (33, 34). When such cells were infected with retroviruses expressing Mad1 (see Materials and Methods), we reproducibly observed an 80% reduction in the number of soft agar colonies compared with cells that were either mock infected or infected with a retrovirus lacking cDNA inserts (Fig. 1A). In addition, we noted that colonies emerging from the cell populations infected with the *mad1*-expressing retrovirus were greatly reduced in size, suggesting an overall reduction in growth rate or a delayed response to ligand (see below and Fig. 3).

To test if the capacity of Mad to suppress cell growth is associated with its interactions with Max and mSin3, we examined the ability of two mutant Mad1 proteins to inhibit the CSF-1-induced response. Mad Δ LZ contains a deletion within the Mad1 leucine zipper that prevents both its binding to Max and its ability to repress transcription, whereas Mad1 L12P/A16P is unable to interact with mSin3 because of proline

FIG. 2. Effect of Mad1 overexpression on the mitogenic response of cells bearing mutant CSF-1R. (A) Colony formation in soft agar of 3T3 cells expressing mutant CSF-1R Y809F following infection with retroviruses expressing the indicated proteins. "Mock" indicates either no virus or control retrovirus lacking cDNA inserts (both controls produced the same results). (B and C) Cells bearing CSF-1R Y809F and stably overexpressing c-Myc (B) or cyclin D1 (C) were infected with retroviruses expressing the indicated proteins. Data are reported as described in the legend for Fig. 1.

protein. These results with the wild-type and mutant Mad1 proteins indicate that the Mad1 transcriptional repression function correlates with an ability to block the mitogenic response to CSF-1.

Mad1 blocks c-Myc and cyclin D1 rescue of CSF-1R Y809F cells. Following CSF-1 treatment, both c-Myc and cyclin D1 are required for proliferation of CSF-1R-expressing 3T3 cells, and ectopic expression of either of the genes that encode these proteins will restore the proliferative potential of cells bearing mutant CSF-1R Y809F (32, 34). To further examine the negative effects of Mad1 on CSF-1-induced cell cycle progression, we ectopically expressed Mad1 in cells bearing mutant CSF-1R Y809F. Alone, neither wild-type nor mutant Mad1 rescued signaling through the receptor mutant (Fig. 2A), demonstrating, as expected, that Mad1 cannot substitute for Myc or cyclin D1. To determine whether Mad1 could block rescue of the CSF-1R Y809F cells by c-Myc and cyclin D1, we established clonal cell lines expressing combinations of the genes that encode these proteins and infected them with either wild-type or mutant Mad1 retroviruses. As shown in Fig. 2B, wild-type Mad1, but not the Mad1 Δ LZ mutant, inhibited the ability of c-Myc to reconstitute CSF-1 mitogenicity. Similarly, wild-type Mad1 blocked cyclin D1 rescue of the cells, whereas both the Mad1 Δ LZ and Mad1 L12P/A16P mutants were without significant effect (Fig. 2C). Therefore, Mad1 can potentially antagonize c-Myc even when the latter is constitutively expressed. Moreover, the ability of Mad1 to prevent rescue by cyclin D1 underscores the dependency of cyclin D1 on endogenous c-Myc function.

Absence of wild-type Mad1 protein expression in proliferating cells. Our results suggest that ectopically expressed wild-type Mad1 effectively blocks the proliferative response of the CSF-1R-bearing cells. If so, we would expect that cells consti-

substitutions at Leu-12 and Ala-16 within the Sin interaction domain (references 4, 19, and 20 and data not shown). In contrast to the results obtained with wild-type Mad1, infection of receptor-bearing cells with retroviruses encoding either Mad1 mutant did not interfere with their response to CSF-1 (Fig. 1A). To ensure that the lack of growth inhibition was not due to a failure of the cells to express the mutant Mad1 proteins, we employed a previously characterized Mad antibody to assess Mad1 protein synthesis in the infected cells. In an earlier study we had demonstrated that this antibody immunoprecipitated the 35-kDa Mad1 protein (2). Analysis of anti-Mad1 immunoprecipitates from cells infected in parallel with each of these retroviruses confirmed that wild-type and mutant Mad1 proteins were ectopically expressed at high levels (Fig. 1B). As expected, uninfected cells did not synthesize detectable Mad1

FIG. 3. Colony formation in semisolid medium. NIH 3T3 cells expressing wild-type CSF-1R were infected with a retroviral vector lacking an insert (mock) or a retrovirus expressing Mad1, Mad1 Δ LZ, or Mad1 L12P/A16P and cloned 72 h later in semisolid medium containing purified recombinant human CSF-1. No colonies were observed in the absence of the growth factor. Photographs were taken 17 days following plating. Magnification, $\times 26$.

tuting the relatively few colonies that appear following infection with Mad1 retroviruses would express little or no Mad1 protein. We noted earlier that colonies from both the CSF-1-treated cells and the Mad1 retrovirus-infected cells were significantly reduced in size and number compared with colonies from mock-infected or mutant Mad1 retrovirus-infected cells (Fig. 3). Analysis of anti-Mad1 immunoprecipitates of cells grown from three randomly selected CSF-1-induced colonies revealed that none of the proliferating cell populations generated from colonies transfected with wild-type Mad1 expressed detectable 35-kDa Mad1 protein (Fig. 4). By contrast, cells from Mad1 L12P/A16P colonies synthesized either very low or high levels of mutant Mad1 protein. Similar results were obtained for cells ectopically expressing cyclin D1. Here, colony-forming ability was again blocked by infection with wild-type Mad1 but not with Mad1 L12P/A16P retrovirus. Again, the cells derived from the wild-type Mad1 retrovirus-infected colonies did not express Mad1 protein, while the mutant Mad1 retrovirus-infected colonies expressed variable levels of mutant Mad1 protein. These findings are consistent with the idea that wild-type Mad1 inhibits cell proliferation in this system and that cells that do proliferate either have downregulated Mad1 expression or do not contain the transfected *mad1* gene. The detection of variable levels of the mutant Mad1 protein suggests that proliferation is most likely not significantly affected by expression of the mutant protein.

Mad1 induces a cell cycle block. To determine whether Mad1 blocks cell proliferation in a specific phase of the cell cycle, we transfected a Mad1 expression vector that also encoded the T-cell coreceptor CD8 into proliferating NIH 3T3 cells. The DNA content of transfected (CD8-positive) and nontransfected (CD8-negative) cells from the same culture dish was measured by dual-laser flow cytometry 48 h after

transfection in five separate experiments, as described in Materials and Methods (Fig. 5). Compared with a vector encoding CD8 alone, the Mad1-containing vector reproducibly induced a significant increase in the fraction of G_1 phase cells (2N DNA content) with concomitant loss of those in the S phase (DNA content between 2N and 4N) and the G_2/M phase (4N DNA). Accumulation of cells in G_1 with Mad1 was comparable to that observed with two G_1 cyclin-dependent kinase-4 inhibitors, p16^{INK4a} and p19^{INK4d} (references 17 and 29 and data not shown). By contrast, ectopic expression of both Mad1 mutants resulted in only a marginal increase in the fraction of G_1 phase cells relative to that obtained with the control vector (Fig. 5 and Table 1). The fact that the mutants had some effect on progression is not completely unexpected. Mad1 L12P/A16P may still possess residual binding activity for mSin3 or complete with Myc for binding to target sites. Likewise Mad1 Δ LZ may interact weakly with Max in vivo or possibly compete with endogenous Myc for limiting factors.

DISCUSSION

The proteins of the Mad family, comprising Mad1, Mxi1, Mad3, and Mad4, have been suggested to functionally antagonize the activities of the Myc protein family (1, 3, 19). This model was based largely on three properties, initially defined for Mad1: (i) the ability to compete with Myc for binding to Max, (ii) the ability of Mad:Max heterodimers to compete with Myc:Max heterodimers for specific binding to DNA, and (iii) the opposing transcriptional activities of Mad1 and Myc (3). Furthermore, the induction of Mad1 during differentiation of a wide variety of cell types (2, 10, 19, 22, 39) during a period when Myc is known to be downregulated is also suggestive of antagonistic functions for Mad1 and Myc. Mad3 and Mad4

FIG. 4. Expression of Mad1 proteins in colonies generated from CSF-1R-expressing 3T3 cells. Three randomly selected colonies derived from the indicated cells were expanded and labeled with [³⁵S]methionine. Cell lysates were immunoprecipitated with anti-Mad1 antibody as described in Materials and Methods.

FIG. 5. Effect of Mad1 overexpression on cell cycle progression. DNA content of NIH 3T3 cells transfected with a vector encoding mouse CD8 alone or together with different Mad1 cDNAs is shown. Histograms display DNA content of cells having CD8-fluorescein isothiocyanate fluorescence greater than the background level obtained with an isotype-matched control antibody. The percentages of cells within the G_1 , S, and G_2/M phases of the cell cycle were determined by analysis of the single-parameter DNA histogram with the computer program ModFit. wt, wild type.

have also been shown to interact with Max and act as transcriptional repressors, and all four proteins can block the co-transformation of primary rat embryo fibroblasts by Myc and Ras (7, 19–21, 39). Here we present evidence that ectopic expression of Mad1 can block the proliferative response of fibroblasts to specific mitogenic signals in a manner that appears to be dependent on its transcriptional repression function.

Although c-Myc is rapidly induced as cells enter the division cycle from quiescence, its expression throughout the proliferative cell division cycle is invariant (15, 38). Functions of *c-myc* are necessary and rate limiting for the G_0 -to-S phase transition (11a, 16, 32, 34), but a role for c-Myc in governing progression through later phases of the cycle has not been ruled out. Indeed, several reports have implicated c-Myc in the G_2/M progression. Increased c-Myc phosphorylation and transcriptional activity have been found during the G_2/M phase of the cell cycle (24, 36) but have not been shown to be required for G_2/M progression. Others have argued that the ability of *c-myc* to overcome an S phase block in epidermal growth factor-stimulated BAF3 pro-B cells reconstituted with epidermal growth factor receptors connotes a physiologic requirement for c-Myc in cells that have exited G_1 (37). However, the latter findings could reflect the existence of underlying checkpoint controls that limit S phase progression under circumstances in which G_1 events are improperly executed. The fact that Mad1 induces G_1 arrest without obviously perturbing S phase or G_2/M progression suggests that c-Myc's positive transcriptional

role during the cell cycle is limited to the G_1 interval. This assumes that Mad1 and Myc act on the same set of target genes. Cyclin D1 is also rate limiting for G_1 progression (5, 30, 31, 41), and while expressed throughout the cell cycle as long as cells are mitogenically stimulated, it is required only during mid to late G_1 phase (5, 30). Inhibition of proliferation with cyclin D1-rescued cells was also obtained with a dominant-negative c-Myc mutant in lieu of Mad1 (34), reinforcing the notion that the observed effects of Mad1 are mediated via c-Myc antagonism. The ability of Mad1 to suppress CSF-1-dependent growth of cyclin D1-rescued cells also argues that cyclin D1 does not simply function "downstream" of c-Myc but instead depends on c-Myc during this portion of G_1 . Nonetheless, our results, while consistent with the idea that Mad1 directly opposes the function of c-Myc, do not provide proof of the model. The inhibition of cell cycle progression by Mad1 could be due to effects unrelated to direct antagonism with Myc. Validation of the model awaits identification of the critical target genes for Myc and Mad1.

Mad1 is expressed at low or undetectable levels during cell proliferation but is induced in many cell types during differentiation (2, 10, 18, 22, 39). In contrast, ectopically expressed Myc blocks differentiation by precluding a G_0/G_1 arrest (13). The ability of Mad1 to arrest cells in G_1 might provide a mechanism for their exit from the cell cycle during differentiation. In this regard, it is interesting that the cyclin-dependent kinase inhibitor p21 is also induced during terminal differentiation (14, 27), and it may ultimately prove that there is a more direct link between the actions of Mad1 and the induction of cyclin-dependent kinase inhibitors, as has been demonstrated with p53 (12, 40). In contrast to Mad1 expression in postmitotic cells, Mxi1 and Mad3 appear to be present in proliferating cells and might instead function to modulate c-Myc-driven cell cycle progression (19, 22, 30a, 42). Our results are in general agreement with those of Chen et al. (9), who reported that ectopic expression of *mad* in some human tumors also leads to a growth arrest in G_1 (9). It would therefore be worth exploring the use of Mad family proteins to selectively inhibit the growth of tumor cells whose oncogenic phenotype derives from a block to differentiation through deregulation of Myc family genes.

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TABLE 1. Effect of Mad1 overexpression on cell cycle progression

Transfected-gene product ^a	No. of expts	% Cells in cell cycle phase (mean $\pm$ SD)		
		G_0/G_1	S	G_2/M
CD8 only	5	49.5 $\pm$ 7.2	33.6 $\pm$ 6.7	16.9 $\pm$ 1.6
Mad1 (wt)	6	75.0 $\pm$ 6.3	15.5 $\pm$ 4.6	9.5 $\pm$ 3.6
Mad1 Δ LZ	3	61.7 $\pm$ 2.8	22.0 $\pm$ 2.7	16.3 $\pm$ 1.3
Mad1 L12P/A16P	3	58.7 $\pm$ 0.9	26.1 $\pm$ 1.8	15.3 $\pm$ 1.7

^a Mad1 genes were transfected with CD8. wt, wild type.

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