Increased Mitotic Phosphorylation of Histone H3 Attributable to AIM-1/Aurora-B Overexpression Contributes to Chromosome Number Instability[1]

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ABSTRACT

Phosphorylation of histone H3 at Ser-10 is required for maintenance of proper chromosome dynamics during mitosis. AIM-1, a mammalian Ipl1/aurora kinase involved in H3 phosphorylation, is transcriptionally overexpressed in many tumor cell lines. Increased expression of the *AIM-1* gene has been observed in human colorectal tumors of advanced grade and stage. Here we report that forced exogenous overexpression of AIM-1 in Chinese hamster embryo cells causes increased mitotic Ser-10 phosphorylation with concomitant induction of lagging chromosomes during mitosis. Lagging chromosomes could also be induced by transfection with mutated histone H3 (S10E), which is thought to maintain Ser-10 in the phosphorylated state. In the present study, chromosome number instability and increased tumor invasiveness were noted in constitutively AIM-1-overexpressing cells *in vivo*. Increased mitotic Ser-10 phosphorylation was also observed in various colorectal tumor cells with high AIM-1 expression levels. These data suggest that increased H3 histone phosphorylation as a result of AIM-1 overexpression is a major precipitating factor of chromosome instability and, thus, may play a role in carcinogenesis.

INTRODUCTION

Subversion of the mechanisms responsible for maintaining ploidy, the normal balance of chromosomes, is a common feature of tumor cells (1, 2). The gain or loss of chromosomes has long been recognized as an important step in converting normal diploid cells into neoplastic aneuploid cells during multistep carcinogenesis (3–6). Loss of mitotic regulation is believed to be one way in which unstable chromosome number phenotypes develop and is thought to create abnormal nuclear morphology in cancer cells (7–11). Among the currently known mitotic subversion mechanisms, a defect in the genes required for spindle checkpoint control may lead to generation of CIN,[3] which is distinguished from MIN when classifying tumors (9). CIN and MIN are genetically unstable phenotypes that manifest at two distinct levels. CIN is observed at the chromosome level and involves loss or gain of chromosomes or large portions of chromosomes, and MIN is observed at the nucleotide level, resulting in base substitutions, deletions, or insertion of a few nucleotides. Most cancers are classified as either CIN or MIN. Aneuploidy is commonly observed, even in tumor cells with normal spindle checkpoint control. The MIN and CIN classifications may reflect only the convenience by which these manifestations can be identified, although both phenotypes may

promote cancer progression as a result of genomic instability. That is, clinically, distinguishing between MIN and CIN may have little significance. Therefore, we need to look beyond the MIN and CIN classification system toward the primary defects responsible for producing aneuploidy.

AIM-1 belongs to the A-kinase subfamily, also called the aurora kinase family, and at least three related kinases, including AIM-1 (Aurora-B), STK15 (Aurora-A), and STK13 (Aurora-C), exist in mammals (12, 13). All three mammalian members of this subfamily are overexpressed in human cancer cells. STK15 is overexpressed in half of all colorectal and breast cancers, and its gene has been mapped to 20q13 and amplified (14–17). STK15 is predominantly localized to centrosomes (15, 16, 18). Overexpression of STK15 is thought to relate to multiple centrosome production (16). STK13 is highly expressed in the testis but is also overexpressed in half of all colorectal cancers (19). Although STK13 has not yet been studied extensively, this protein likely associates with centrosomes (20). Overexpression of AIM-1, on the other hand, is seen in many human cancers (21, 22). Expression levels of AIM-1 increase as a function of Dukes' stage in colorectal tumors (22). AIM-1 and its corresponding proteins, *Caenorhabditis elegans* AIR-2, *Xenopus laevis* AIRK2, and *Drosophila* IAL, are localized in the spindle midzone and midbody during mitosis and have roles in cytokinesis (23–26). Furthermore, these kinases were found recently to be responsible for mitotic phosphorylation of histone H3 at Ser-10 (27–29), suggesting that AIM-1 and its related kinases (Aurora-B kinases) are functional homologues of *Saccharomyces cerevisiae* Ipl1. Mitotic phosphorylation of histone H3 is a known requirement for proper sister chromatid segregation into daughter cells (30–32). Moreover, double-stranded RNA interference analysis has revealed that Aurora-B plays a critical role in chromosome segregation, as well as cytokinesis (13).

We here report that AIM-1 overexpression induces increased phosphorylation of histone H3 at Ser-10 during mitosis in CHE cells, and that the forcedly exogenously overexpressing cells produce aneuploid cells capable of forming aggressive tumors in nude mice. Importantly, increased mitotic phosphorylation is observed in both CIN and MIN cells from human colorectal tumors. These results suggest that AIM-1 overexpression is important in aneuploidy formation during carcinogenesis.

MATERIALS AND METHODS

Cell Lines. The culture conditions of all cell lines used in this experiment have been described previously (21). CHE diploid fibroblasts (CHE cells) derived from whole embryos were established, and various cell lines with normal TP53 status and mutated TP53 status were subcloned (33). Initially, we used a cell line with normal TP53 (designated clone A1/p60/clone 4 with a modal chromosome number of 22), and the cells were transfected with AIM-1 expression vectors. However, cell growth was suppressed by AIM-1 expression, and no stable AIM-1-expressing clones were isolated. Then, we switched to a cell line with G245S mutations in both alleles of the *TP53* gene. This cell line was confirmed to be defective in TP53-dependent p21 induction after X-ray irradiation. This cell line, designated clone A1/p60/clone 3, had one marker chromosome [t(1q;9)] and a modal chromosome number of 23. The

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cells were tumorigenic after being injected into nude mice (10^7 cells/animal), although the incidence was not high (4 of 10), and a rather long latency period was required (>50 days). Furthermore, the tumors that developed were not pathologically aggressive. Human colorectal tumor cell lines were also used here, and these cell lines were obtained from American Type Culture Collection.

Plasmids. The construction of FLAG-tagged AIM-1 constructs has been described previously (23). Human histone H3 cDNA was cloned from a cDNA library constructed from human colorectal cancer SW480 cells (34). A mutant of H3 (S10E) was generated by site-directed mutagenesis using PCR. The cDNAs were subcloned into a pcDNA3.1-His/Xpress vector (Invitrogen). The RSV promoter-regulated pRSV-β-galactosidase encoding *Escherichia coli* β-galactosidase was used as a reporter construct for transfection.

DNA Transfection. Transfection was performed using LipofectAMINE (Life Technologies, Inc.). Exponentially growing cells were transfected with an appropriate plasmid construct. The transfected cells were cultured for 24 h and then treated with (N) or without (E) 0.2 μg/ml nocodazole for 18 h. In the NR experiments, the cells were washed to remove nocodazole, followed by incubation for 1 h. Then, the cells were lysed for Western blotting or fixed for immunostaining.

Western Blotting and Densitometric Analysis. Cells were extracted for Western blotting as described previously (23). Boiled and sonicated lysate was loaded onto a polyacrylamide gel. The protein was electroblotted onto a Durapore filter (Millipore) and incubated with anti-Ser-10 phosphorylated H3 histone rabbit polyclonal antibody (Upstate), anti-histone H3 goat polyclonal antibody (Santa Cruz Biotechnology), anti-FLAG M5 monoclonal antibody (Eastman Kodak), or anti-AIM-1 rabbit polyclonal antibody (23). Anti-Ser-28 phosphorylated H3 histone rabbit polyclonal antibody (Upstate) was also used. The immunoblot was developed with ECL reagent (Amersham). Quantification of phosphorylated H3 histone at Ser-10 was performed by densitometric analysis using the lumino-imaging analyzer LAS-1000 (Fuji Film). The amount of phosphorylated H3 histone was expressed by calibration of the amount of H3 histone in the filter. In experiments involving the colorectal cancer cell lines, the data obtained were further calibrated by the mitotic index because CIN and MIN cells have different mitotic indexes after treatment with nocodazole.

Cell Immunostaining and β-Galactosidase Cytochemistry. The cell fixation procedure was described previously (23). Samples were immunostained with the primary antibody [either anti-FLAG M5 monoclonal antibody (Kodak), anti-AIM-1 rabbit polyclonal antibody (23), or anti-Xpress mouse monoclonal antibody (*Invitrogen*)], after which they were stained with the secondary antibody [either Rhodamine-conjugated antimouse IgG or fluorescein-conjugated antirabbit IgG (Chemicon)]. For DNA staining, DAPI was used. The stained cells were observed with a Zeiss LSM-510. To quantitate the number of affected cells after transfection, expression of β-galactosidase was visualized by a method described previously (23), followed by Giemsa staining. The β-galactosidase-positive cells were analyzed by microscopy.

Chromosome Analysis. Cultured cells were treated with Colcemid (0.04 μg/ml for 4 h). Cells were then collected, and the suspended cells were treated with hypotonic KCl and fixed with Carnoy's solution (35). Chromosome spreading and Giemsa staining were performed by conventional methods. The samples were then analyzed by microscopy.

***In Vivo* Tumor Growth.** In accordance with a previous report (36), cells were s.c. injected into BALB/c *nu/nu* mice (10^7 cells/mouse), 6 weeks of age. The animals (4–6 mice/group) were observed, and tumor diameters were measured to estimate tumor weight. Tumor weight was calculated using the following formula: weight (mg) = a^2b^2, where *a* (mm) is the major axis and *b* (mm) is the minor axis. Actually, in our experiment, tumor weights in mice that received injections s.c. with Lewis lung carcinoma cells (3LL cells) were closely correlated with the calculated predicted values (correlation coefficient, 0.761; *n* = 31). The animals were sacrificed after 91 days to measure actual final tumor weights and to make pathological observations.

RESULTS

Chromosome Lagging Is Concomitant with Increased Mitotic Phosphorylation of Histone H3 at Ser-10 in AIM-1-transfected Cells. Phosphorylation of histone H3 at Ser-10 occurs during mitosis in a diverse range of eukaryotes and is required for proper chromo-

some condensation and segregation (30–32). In higher eukaryotes, Aurora-B kinases are thought to be responsible for this phosphorylation (27–29). Therefore, it is reasonable to suggest that, if mammalian Aurora-B kinase AIM-1 were overexpressed in cultured cells, mitotic phosphorylation might increase. Consequently, chromosome dynamics might be affected. To test this possibility, FLAG-tagged AIM-1 was expressed in CHE cells, and the phosphorylation status and chromosome segregation pattern of these cells were examined by Western blotting with anti-phospho-histone H3 (Ser-10) rabbit polyclonal antibody. Immunofluorescence staining with anti-FLAG M5 monoclonal antibody and DAPI, respectively, was also performed. As shown in Fig. 1*A*, increased mitotic phosphorylation was seen with AIM-1 overexpression, whereas phosphorylation was suppressed after transfection of a dominant-inactive AIM-1-K/R (23, 34). Lagging chromosomes and chromosome bridges were observed in AIM-1-overexpressing mitotic cells (Fig. 1*B*). In addition, multinuclear cells were found in AIM-1-overexpressing interphase cells, as reported previously (Refs. 21, 34; Fig. 1*B*). When observed after 42 h of transfection, immunofluorescence studies revealed that 66% of FLAG-tagged AIM-1-overexpressing mitotic cells had abnormal chromosomes, manifesting as amorphous lagging chromosomes or chromosome bridges, and that 26% of overexpressing interphase cells were multinuclear (Fig. 1*C*). This indicates that AIM-1 overexpression triggers chromosome number instability, as well as multinuclearity, concomitant with an increased mitotic phosphorylation of histone H3 at Ser-10.

Mutant H3 (S10E), a Constitutively Expressed Phosphorylated Form of Ser-10, Induces Chromosome Lagging. To further probe the role of increased mitotic phosphorylation of histone H3 at Ser-10 with regard to chromosome segregation, we generated a mutant H3 expression plasmid in which the Ser-10 site was changed to glutamic acid (S10E). This plasmid was designed to mimic the stable phosphorylated form of Ser-10 (37, 38). Ectopic expression of the Xpress-tagged mutant H3 induced chromosome lagging and chromosome bridging during anaphase of mitosis (Fig. 2*A*). When observed after 43 h of transfection, 58% of Xpress-tagged mutant H3 (S10E)-overexpressing mitotic cells had abnormal chromosomes, whereas mitotic cells expressing wild-type H3 did not, compared with control cells lacking exogenous H3 expression (Fig. 2*B*). Both wild-type and mutant H3 protein were found in cell nuclei during interphase and were localized to chromosomes in mitotic cells (Fig. 2*A*). Thus, it appears that exogenous H3 protein was incorporated into the chromatin of the cells. These results indicate that the S10E mutant may act in a dominant-negative fashion to affect chromosome segregation in CHE cells, which further suggests that H3 phosphorylation might play a critical role in chromosome separation.

Stable Transfectants Engineered to Overexpress AIM-1 Undergo Extensive Phosphorylation during Mitosis and Have an Unstable Chromosome Number. To test the importance of AIM-1 overexpression in generating chromosome number instability and possibly carcinogenesis, constitutively AIM-1-overexpressing stable clones were isolated from CHE cells by transfection with a pcDNA3-FLAG-AIM-1 expression plasmid carrying a G418 resistance marker. Stable transfectants were then characterized, and G418-resistant transfected clones (53 independent clones) were examined for AIM-1 expression using anti-FLAG M5 monoclonal antibody. Five clones with different AIM-1 expression levels were chosen. AIM-1-transfected cells (pooled clones and five independent clones), empty vector-transfected cells (pooled clones and two independent clones), and MOCK-transfected cells were subsequently cultured for five passages (35 days). H3 phosphorylation levels, as well as H3 histone, AIM-1, and FLAG-AIM-1 expression levels were examined in each culture using anti-phospho-histone H3 (Ser-10) rabbit polyclonal
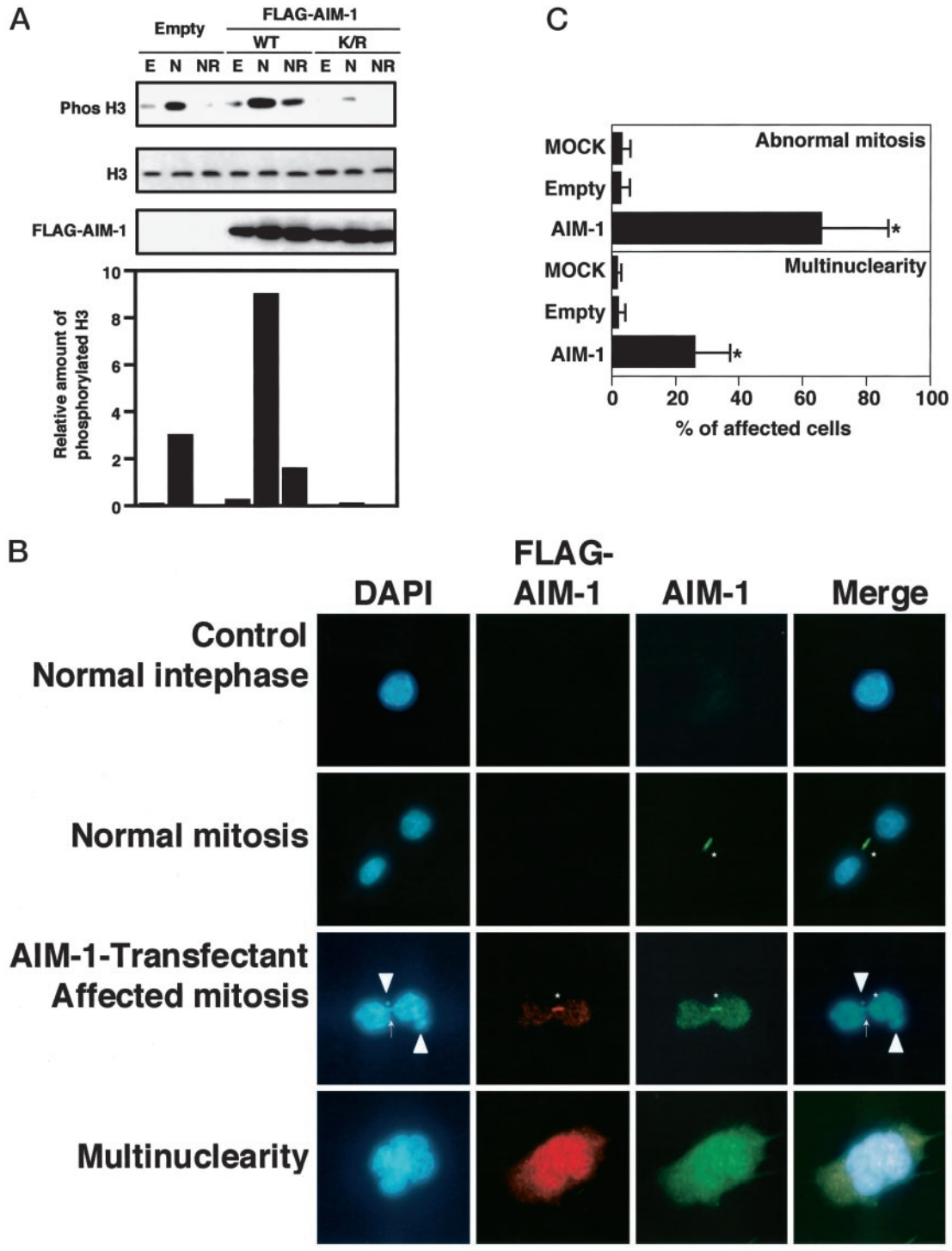
Fig. 1. Effects of transient expression of AIM-1 on H3 histone phosphorylation and chromosome segregation. *A,* mitotic phosphorylation of H3 histone at Ser-10 induced by AIM-1. Exponentially growing CHE cells were transfected with pcDNA3-FLAG-AIM-1 encoding FLAG-tagged wild-type AIM-1 (*WT*), pcDNA3-FLAG-AIM-1 encoding FLAG-tagged kinase-inactive AIM-1 (*K/R*), or empty vector (*Empty*). The transfected cells were cultured for 24 h and then treated with (*N*) or without (*E*) 0.2 μg/ml nocodazole for 18 h. In the NR experiments (*NR*), the cells were washed to remove nocodazole, followed by incubation for 1 h. The cell lysate was immunoblotted with anti-Ser-10 phosphorylated H3 histone rabbit polyclonal antibody (*Phos H3*), anti-histone H3 goat polyclonal antibody (*H3*), and anti-FLAG M5 monoclonal antibody (*FLAG-AIM-1*). Quantification of the relative amount of phosphorylated H3 histone at Ser-10 was performed by densitometric analysis using an immunoblotting filter and calibrating results according to the total amount of H3 histone in the filter. *B,* representative immunofluorescence microscopic images from control CHE cells in normal interphase (*first lane*) or in normal mitosis (cytokinesis; *second lane*), and from AIM-1-transfected CHE cells with mitotic lagging chromosomes and chromosome bridging (*third lane*), as well as abnormal nuclei (*fourth lane*). Control and AIM-1-transfected cells were fixed 43 h after transfection and were stained with DAPI for DNA staining, and anti-FLAG M5 monoclonal antibody and anti-AIM-1 rabbit polyclonal antibody. The white arrowheads indicate lagging chromosomes in affected mitotic cells. *White arrows,* chromosome bridging in affected mitosis. ∗, the midbody during cytokinesis. *Scale bar,* 20 μm. *C,* quantitative data demonstrating the effect of AIM-1 overexpression on cell division. Wild-type AIM-1 [pcDNA3-FLAG-AIM-1(WT)] or empty vector were cotransfected with a pRSV-β-galactosidase reporter construct into CHE cells. In the control experiments, MOCK transfection was performed. The cells were fixed for 43 h after transfection, stained with Giemsa, and analyzed by microscopy. Expression of β-galactosidase was visualized as described in "Materials and Methods." To quantitate abnormal mitosis with chromosome lagging and bridge formation in transfected cells, the number of β-galactosidase-positive mitotic cells (of 50 mitotic cells) was counted, and the percentage of affected mitotic cells was calculated. To quantitate the proportion of multinuclear cells, the number of β-galactosidase-positive interphase cells (of 100 cells) was counted, and the percentage of cells with multiple nuclei was calculated. In MOCK-transfected cells, the proportion of affected mitotic cells or the proportion of multinuclear interphase cells were calculated. The data were obtained from three independent experiments. *Columns,* means; *bars,* SD. ∗, $P < 0.05$ compared with *MOCK* or *Empty* vectors by the two-sample *t* test with Welch's correction (two-tailed).
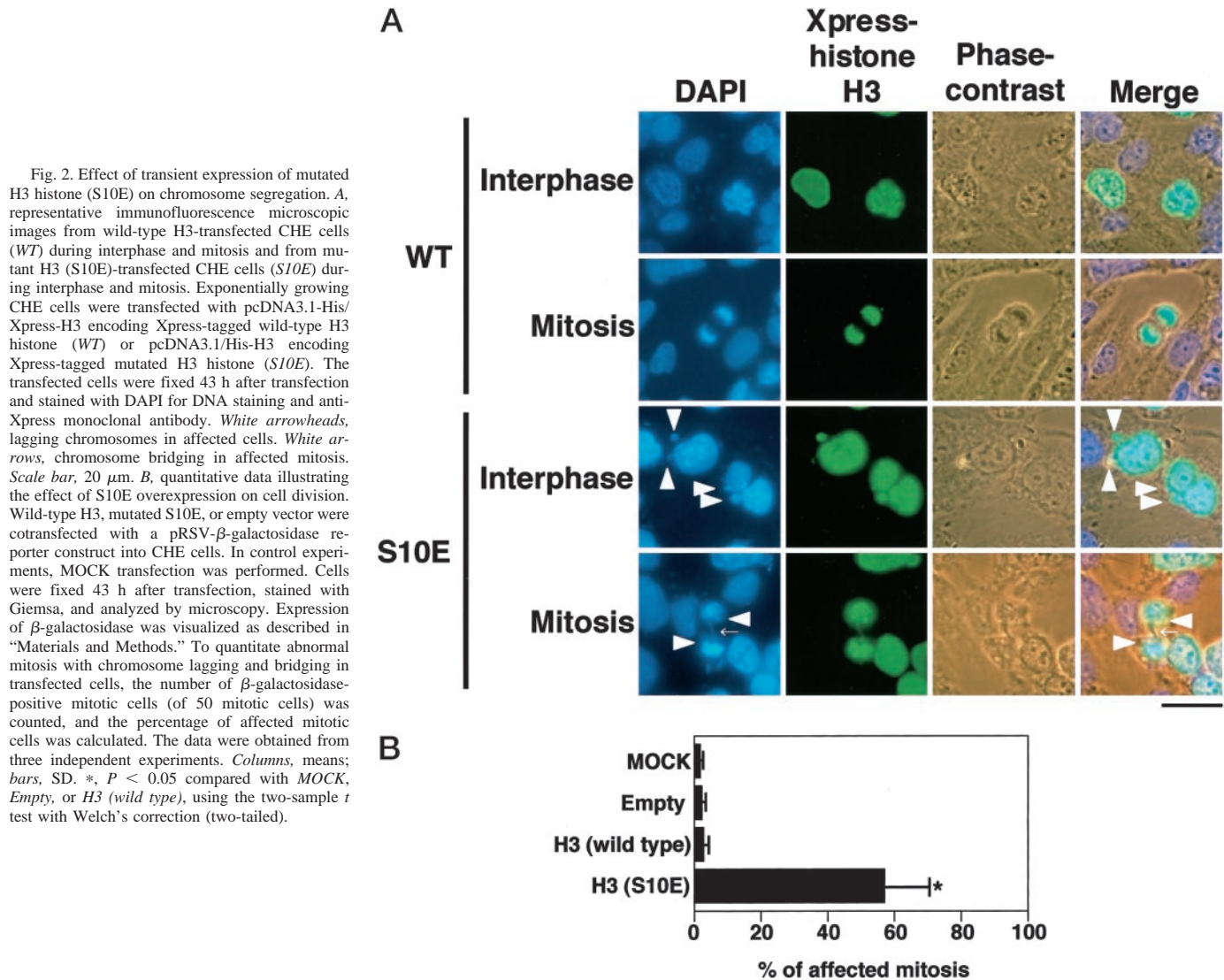
Fig. 2. Effect of transient expression of mutated H3 histone (S10E) on chromosome segregation. *A,* representative immunofluorescence microscopic images from wild-type H3-transfected CHE cells (*WT*) during interphase and mitosis and from mutant H3 (S10E)-transfected CHE cells (*S10E*) during interphase and mitosis. Exponentially growing CHE cells were transfected with pcDNA3.1-His/Xpress-H3 encoding Xpress-tagged wild-type H3 histone (*WT*) or pcDNA3.1/His-H3 encoding Xpress-tagged mutated H3 histone (*S10E*). The transfected cells were fixed 43 h after transfection and stained with DAPI for DNA staining and anti-Xpress monoclonal antibody. *White arrowheads,* lagging chromosomes in affected cells. *White arrows,* chromosome bridging in affected mitosis. *Scale bar,* 20 μm. *B,* quantitative data illustrating the effect of S10E overexpression on cell division. Wild-type H3, mutated S10E, or empty vector were cotransfected with a pRSV-β-galactosidase reporter construct into CHE cells. In control experiments, MOCK transfection was performed. Cells were fixed 43 h after transfection, stained with Giemsa, and analyzed by microscopy. Expression of β-galactosidase was visualized as described in "Materials and Methods." To quantitate abnormal mitosis with chromosome lagging and bridging in transfected cells, the number of β-galactosidase-positive mitotic cells (of 50 mitotic cells) was counted, and the percentage of affected mitotic cells was calculated. The data were obtained from three independent experiments. *Columns,* means; *bars,* SD. ∗, $P < 0.05$ compared with *MOCK, Empty,* or *H3 (wild type)*, using the two-sample *t* test with Welch's correction (two-tailed).

antibody, anti-histone H3 goat polyclonal antibody, anti-AIM-1 rabbit polyclonal antibody (23), and anti-FLAG M5 monoclonal antibody, respectively. As shown in Fig. 3*A*, the expression level of AIM-1 was observed to be periodic, with maximum expression being noted during the G$_2$-M phases. This is consistent with our previous observations (21). The level of Ser-10 phosphorylation in mitotic cells was higher in AIM-1-transfected clones than in control cells or empty vector-transfected clones. (Fig. 3*A*). After removal of nocodazole, the level of phosphorylated H3 was reduced but remained greater in AIM-1-overexpressing cells than in control cells (Fig. 3*A*, *Lanes N* and *NR*). Among the five stable AIM-1-overexpressing clones, mitotic phosphorylation correlated with AIM-1 expression levels (Fig. 3*B*). To examine the effect of AIM-1 overexpression on chromosome number stability, analysis of metaphase chromosome spread was performed after Colcemid treatment (0.04 μg/ml for 4 h). In the parental CHE cells, a marker chromosome [t(1q;9)] with a modal chromosome number of 23 was noted in 32% of cells (Fig. 4*A, CHE*). The chromosome frequency distribution was virtually the same in empty vector-transfected clones and control cells (Fig. 4*A, Empty*). On the other hand, the proportion of AIM-1-overexpressing clones having the same modal chromosome number was reduced, and a diffuse spread in chromosome number was observed (Fig. 4*A, AIM-1*). Moreover, cells with higher AIM-1 expression levels had a tendency toward

increase of near-tetraploid cells with >36 chromosomes (Fig. 4*B*). Thus, it appears that overexpression of AIM-1 leads to aneuploidy, an abnormal balance of chromosomes, via increased mitotic phosphorylation of histone H3 at Ser-10.

AIM-1-overexpressing Stable Transfectants Show an Aggressive Cancerous Phenotype *in Vivo*. The AIM-1-overexpressing stable transfectants examined above were further characterized according to their cancer phenotypes *in vivo*. As shown in Fig. 5*A*, *in vivo* tumor growth rates were greater after s.c. injection in highly AIM-1-overexpressing cells than in low AIM-1-overexpressing cells or empty vector-transfected cells. Ninety-one days after s.c. injection, both incidence of tumorigenicity and tumor weight were greater in AIM-1-overexpressing cells (Fig. 5*B*). Histological observation revealed that tumors from AIM-1-overexpressing cells were more aggressive than tumors occurring in control cell lines (Fig. 5*C*). We noted that a large number of tumor cells produced by injection of AIM-1-overexpressing cells (clones 1, 3, and 5 and pool clones) were aneuploid and multinuclear. Their AIM-1 expression levels were high, even in tumors produced by injection of clone 1 cells with low AIM-1 expression levels (data not shown). Furthermore, pulmonary and liver metastatic nodules were found in tumor-bearing mice injected with pool clones, clones 1, 3, and 5, as well as in those injected with pool clones and clone 1, respectively. However, no metastatic nodules were
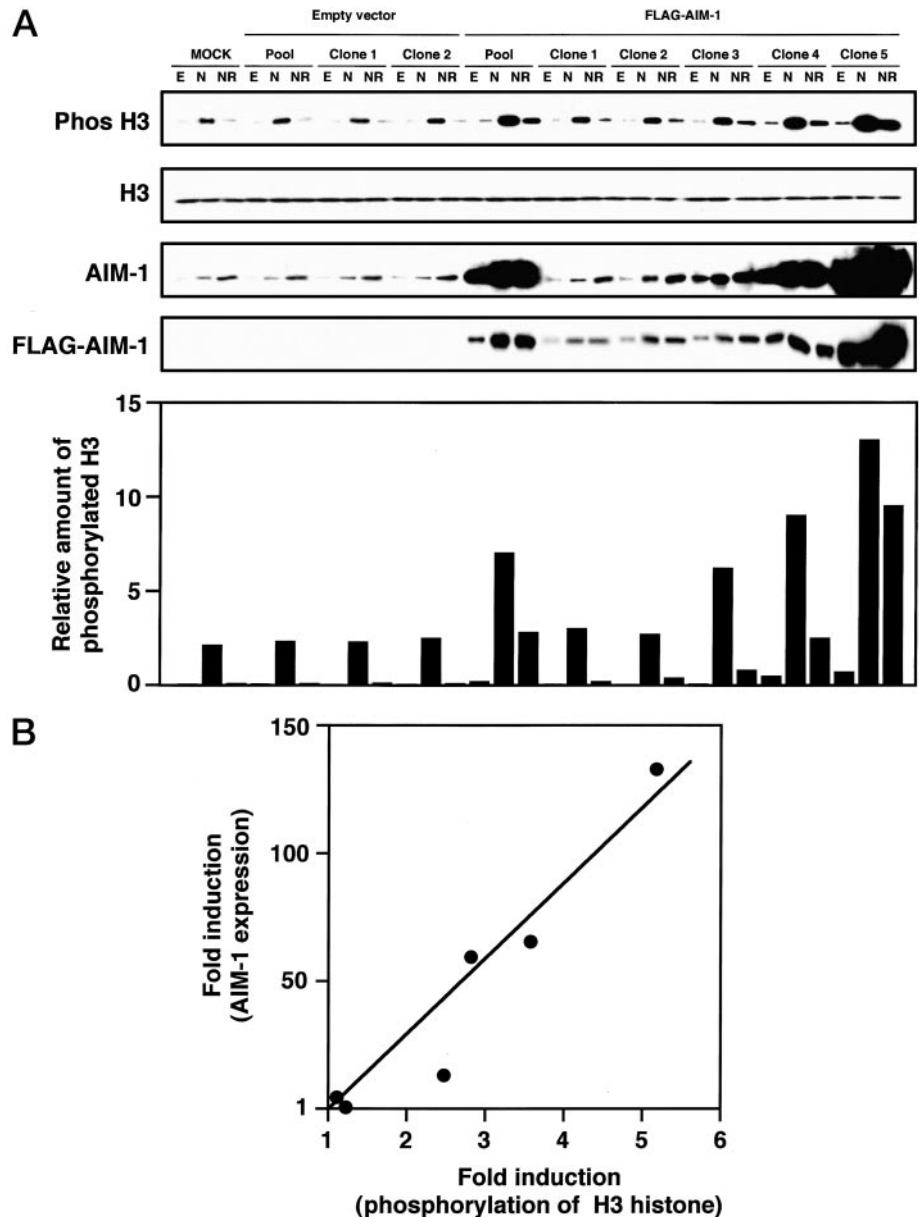
Fig. 3. AIM-1 expression and H3 phosphorylation levels in stable AIM-1-transfectants. *A,* CHE cells were MOCK-transfected or transfected with either pcDNA3-FLAG-AIM-1 (WT) or empty vector. The transfected cells were selected by treatment with G418, and the stable transfectants were obtained. The MOCK-transfected cells were cultured without G418. The G418-resistant clones (53 independent clones) were examined for AIM-1 expression using anti-FLAG M5 monoclonal antibody, and five clones with different AIM-1 expression levels were chosen. The AIM-1-transfected cells (pooled clones and five independent clones), the empty vector-transfected cells (pooled clones and two independent clones), and the MOCK-transfected cells were subsequently cultured for five passages (35 days). The cultured cells were treated with (*N*) or without (*E*) 0.2 μg/ml nocodazole for 18 h. In the NR experiments (*NR*), the cells were washed to remove nocodazole, followed by incubation for 1 h. The cell lysate was immunoblotted with anti-Ser-10 phosphorylated H3 histone rabbit polyclonal antibody (*Phos H3*), anti-histone H3 goat polyclonal antibody (*H3*), anti-AIM-1 rabbit polyclonal antibody (*AIM-1*), and anti-FLAG M5 monoclonal antibody (*FLAG-AIM-1*). Quantification of phosphorylated H3 histone at Ser-10 was performed by densitometric analysis using an immunoblotting filter and calibrating results according to the total amount of H3 histone in the filter. *B,* the inductive effect of mitotic phosphorylation of H3 histone at Ser-10 on AIM-1 expression in stable AIM-1-overexpressing clones. The level of phosphorylated H3 represents the relative amount of AIM-1 transfection *versus* MOCK transfection under nocodazole treatment conditions. A direct relationship was observed when AIM-1 expression was plotted against phosphorylated H3 levels during mitosis (correlation coefficient, 0.924; $n = 6$).

found in tumor-bearing mice injected with empty vector-transfectants or parental CHE cells. Thus, we conclude that AIM-1 overexpression may contribute to cancer progression by producing aneuploidy through increased mitotic phosphorylation of histone H3 at Ser-10.

Increased Mitotic Phosphorylation of Histone H3 at Ser-10 and AIM-1 Overexpression Occur Concurrently in Both CIN- and MIN-classified Human Colorectal Tumor Cell Lines. Because AIM-1 overexpression appears to be the driving force behind production of aneuploidy because of increased mitotic phosphorylation of histone H3 at Ser-10 in CHE cells, we attempted to examine whether increased mitotic phosphorylation leading to aneuploidy might occur in human cancer cells. Human colorectal cancer cells were classified according to the presence of CIN or MIN (9). However, every cell line we could obtain had aneuploidy and multinuclearity, as specified in the American Type Culture Collection catalogue (American Type Culture Collection, Manassas, VA). Furthermore, we confirmed that the cell lines DLD-1, SW48, HCT116, SW620, SW837, SW480, LoVo, and HT-29 show indistinguishable malignancy in transplanted

nude mice.[4] We then theorized that the CIN phenotype, which is known to be associated with a defect of mitotic checkpoint control (39), might not be a major direct cause of aneuploidy but rather may function to promote chromosomal instability via a defect in checkpoint control. If so, aneuploidy in colorectal tumor cells lines might be the result of increased mitotic phosphorylation of H3. As expected, all tumor lines tested showed increased mitotic phosphorylation of H3, as well as overexpression of AIM-1 (Fig. 6*A*). Lower mitotic phosphorylation levels of histone H3 were noted in nocodazole + release-treated CHE cells (Fig. 3*A, Lanes N* and *NR*), but not colorectal cancer cells, compared with nocodazole-treated cells (Fig. 6*A, Lanes N* and *NR*). This suggests that cytokinesis is regulated differently among the two types of cell lines or that they have different nocodazole sensitivities. Fig. 6*B* showed the level of mitotic H3 phosphorylation. Although the mitotic indexes of nocodazole-treated cells differed depending on whether CIN or MIN cell lines were examined because

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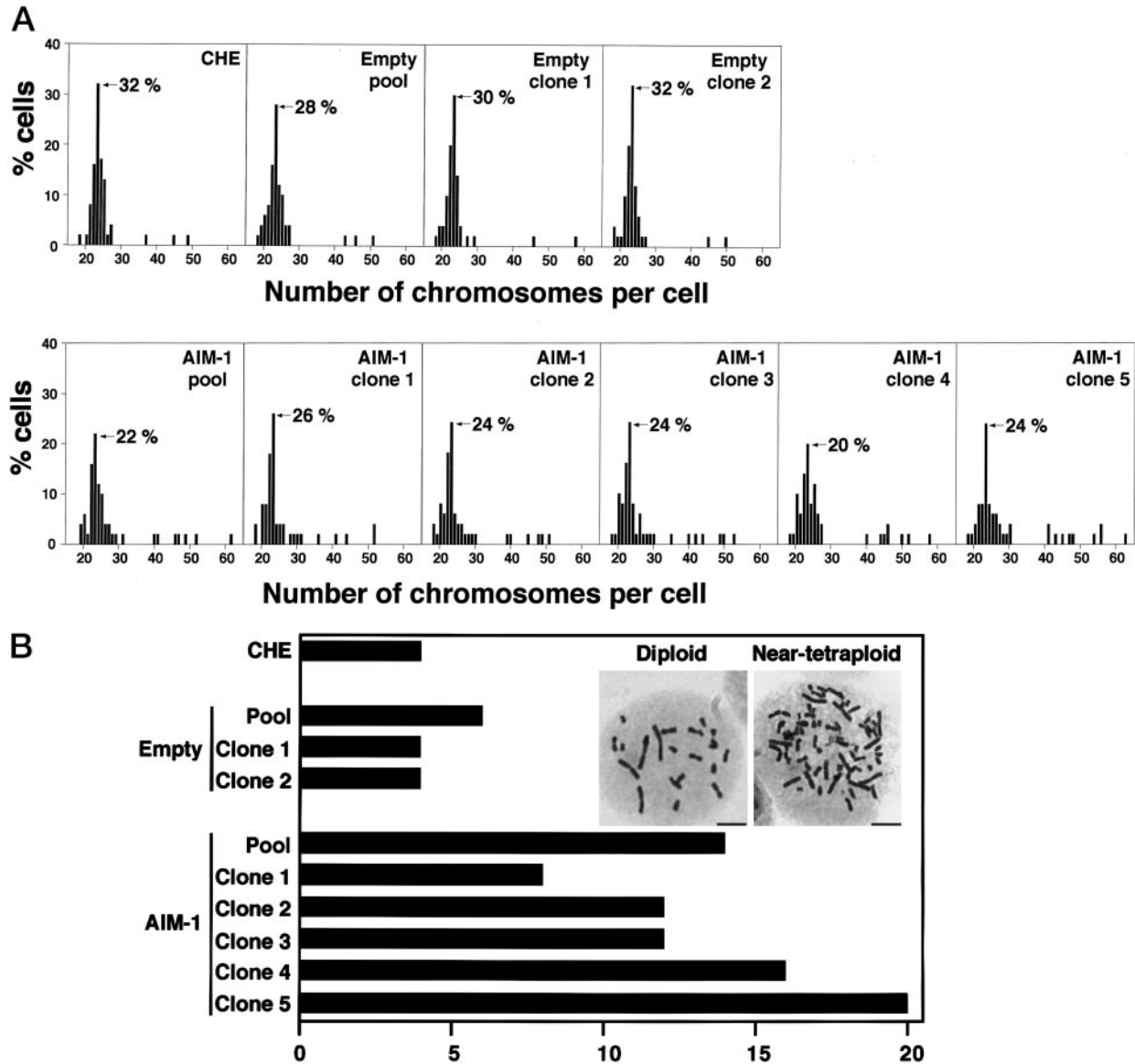[4] T. Ota and M. Tatsuka, unpublished data.

Fig. 4. Effect of AIM-1 overexpression on chromosome number in stable AIM-1 transfectants. *A*, distribution of the number of chromosomes found within cells of different CHE cell lines. The cell lines indicated in Fig. 3 were treated with Colcemid (0.04 μg/ml for 4 h). The chromosomes were visualized by Giemsa staining, and the chromosome number distribution was analyzed. *B*, percentage of near-tetraploid cells (*inset*, representative images of metaphase chromosome spread). The percentage of near-tetraploid cells having >36 chromosomes was calculated from Fig. 2*A*. Typical Giemsa-stained chromosomes of diploid and near-tetraploid cells are shown in the *inset* (*scale bar*, 10 μm).

of the fact that they respond differently to the spindle disruption agent, increased mitotic H3 phosphorylation was consistently observed in all tumor cell lines.

DISCUSSION

AIM-1 Is Responsible for Mitotic H3 Phosphorylation at Ser-10 in Mammals. There are at least three AIM-1-related kinases in mammals, AIM-1 (Aurora-B), STK15 (Aurora-A), and STK13 (Aurora-C), but among these only AIM-1 is capable of phosphorylating histone H3 at Ser-10 during mitosis *in vivo*. Indeed, neither STK15 nor STK13 induce mitotic phosphorylation of histone H3 at Ser-10 after transfection of CHE cells with the expression plasmids pcDNA3-FLAG-STK15 or pcDNA3-FLAG-STK13.[5] Expression of a kinase-negative form of AIM-1 in CHE cells (23, 34) suppresses mitotic phosphorylation of histone H3 at Ser-10. In contrast, expression of kinase-

negative mutants for STK15 and STK13 in CHE cells do not effect phosphorylation.[5] These data indicate that AIM-1 is the only kinase responsible for histone H3 phosphorylation in mammals. In addition, AIM-1 can phosphorylate histone H3 at Ser-28.[5] This may be functionally subsidiary to Ser-10 phosphorylation (40). AIM-1 is thus a major kinase for histone H3 phosphorylation in mitotic cells, and the phosphorylation status of histone H3 may be balanced by AIM-1 kinase activity and PP1 phosphatase activity (27).

Role of Mitotic H3 Phosphorylation at Ser-10. Mitotic phosphorylation of histone H3 at Ser-10 is critical for proper chromosome condensation and segregation. Wei *et al.* (31, 32) have demonstrated abnormal chromosome segregation in a histone H3 mutant unable to undergo Ser-10 phosphorylation because of substitution of Ser-10 with alanine (S10A). Here we observed the same segregation defect in a S10E mutant, which was engineered to mimic the stable phosphorylated form of histone H3 (see Fig. 2). The phosphorylation status of H3 at Ser-10 in turn influences methylation at lysine 9 of histone H3 by SUV39H1 methylase (41). Loss of SUV39H1 function induces

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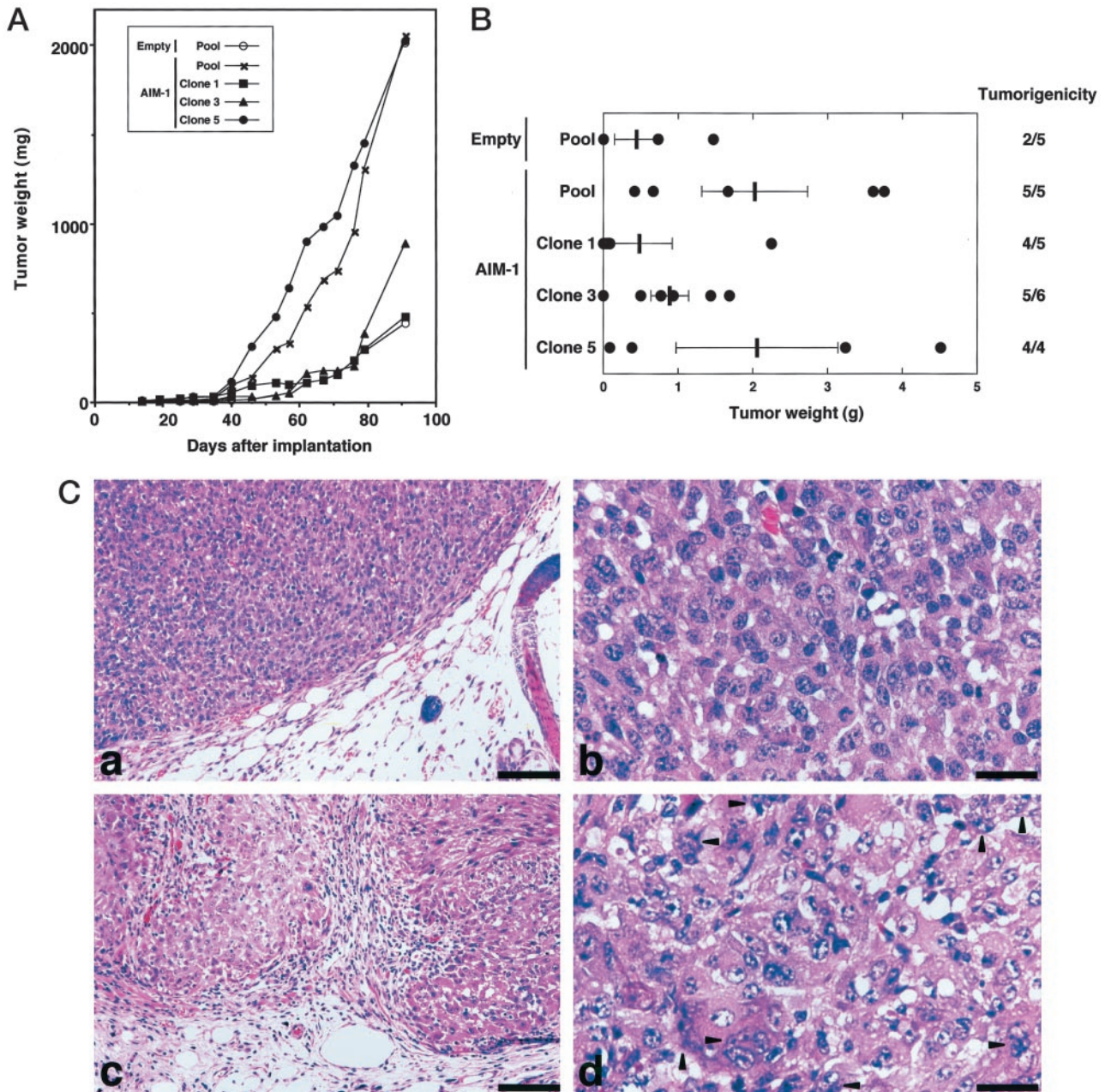[5] S. Suto and M. Tatsuka, unpublished data.

Fig. 5. *In vivo* growth properties and pathological observations of stable AIM-1 transfectants after injection into nude mice. *A,* tumor growth rates after s.c. injection into nude mice. AIM-1-transfected cells (pooled clones and clones 1, 3, and 5) and empty vector-transfected cells (pooled clones) were s.c. injected into 6-week-old BALB/c *nu/nu* mice (10^7 cells/mouse). Tumor diameter was measured, and weight was estimated using a standard method. *B,* tumor weight and tumor incidence 91 days after s.c. injection into nude mice. The injected mice were sacrificed on the 91st day, and their tumor weights were measured. Each *point* represents data obtained from an individual mouse. *Bars,* average ± SE. *C,* representative histology of tumors from pooled empty vector-transfected stable clones (*a* and *b*) and pooled stable clones with AIM-1-overexpression (*c* and *d*). The *black arrowheads* in *d* indicate abnormal nuclei (*i.e.,* micronuclei or multinuclear cells). *Scale bars* of the *left panels,* 100 μm; *scale bars* of the *right panels,* 25 μm.

chromosome segregation errors and concomitant increased H3 phosphorylation at Ser-10 (41). Thus, both substitution of Ser-10 with glutamic acid and AIM-1 overexpression may act to impair H3 methylation. Methylation of H3 histone at lysine 9 by SUV39H1 has also been implicated in recruiting heterochromatin protein 1 to heterochromatin sites, where it mediates gene silencing (42–45). This has led us to believe that chromatin-based regulatory mechanisms may be affected by AIM-1 overexpression and consequently, epigenetic alteration of cancer cells may occur as a result, leading to carcinogenesis. However, there is currently no direct evidence to support this claim.

AIM-1 Overexpression Induces Chromosome Segregation Error, as well as Errors in Cytokinesis. In the present study, we show that AIM-1 overexpression induces increased mitotic H3 phosphorylation at Ser-10. This imbalance in phosphorylation is thought to be one reason why AIM-1 overexpression induces chromosome segregation errors. In addition, AIM-1 overexpression induces the appearance of multinuclear cells because of errors in cytokinesis. This occurs after sister chromatid separation; thus, histone H3 is not involved, rather, AIM-1 may be implicated because we previously established its importance in the onset of cytokinesis (23). Recently, Adams *et al.* (29) have reported that *Drosophila* IAL, which is an AIM-1 counter-
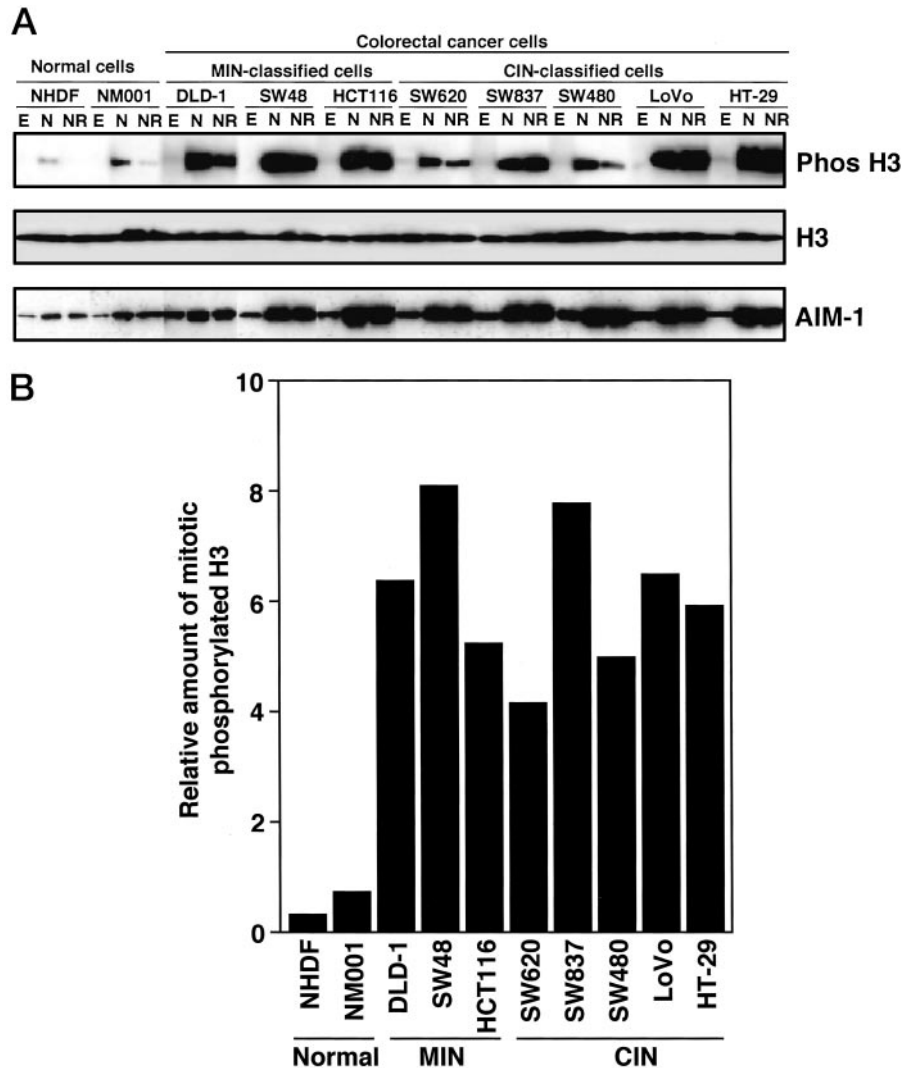
Fig. 6. AIM-1 expression and increases in mitotic phosphorylation of histone H3 at Ser-10 in MIN- and CIN-classified colorectal cancer cell lines. *A,* human colorectal tumor cells and normal control cells were cultured and treated with (*N*) or without (*E*) 0.2 μg/ml nocodazole for 18 h. In the NR experiments (*NR*), the cells were washed to remove nocodazole, followed by incubation for 1 h. The cell lysate was immunoblotted with anti-Ser-10 phosphorylated H3 histone rabbit polyclonal antibody (*Phos H3*), anti-histone H3 goat polyclonal antibody (*H3*), and anti-AIM-1 rabbit polyclonal antibody (*AIM-1*). *B,* quantification of the relative amount of mitotic phosphorylated H3 after normalization using the mitotic index of each cell line. Quantification of phosphorylated H3 histone at Ser-10 was performed by densitometric analysis using an immunoblotting filter after calibrating results according to the total amount of H3 histone in the filter. All quantitative data were normalized according to the mitotic index of each cell line, after which the relative amount of mitotic phosphorylated H3 was calculated. No statistically significant differences with regard to mitotic phosphorylated H3 levels were noted between CIN and MIN.

part, and its binding protein, INCENP, are not required for anaphase A chromosome movement. In addition, they have shown that *Drosophila* IAL and INCENP are not required for correct localization of the kinesis-like protein CHO1/MKLP1 to the midbody at telophase (29). Therefore, the cytokinesis error caused by AIM-1 overexpression may be attributable to increased phosphorylation of an unknown AIM-1 substrate other than histone H3. Although the exact mechanism by which chromosome segregation and cytokinesis errors occur as a result of unbalanced AIM-1 substrate phosphorylation remains to be seen, it is noteworthy that both errors appear to be induced by AIM-1 overexpression and appear to involve different mitotic regulation pathways.

Disturbances in Mitotic Control Attributable to AIM-1 Overexpression: Implications for Carcinogenesis. There are at least three AIM-1-related kinases, termed Aurora kinases, in mammals (12, 13). Significant overexpression of AIM-1 has been observed in many cancer cell lines and several human cancers examined to date (21, 22). Overexpression of STK15 and STK13 has been observed in ~50% of all cancer cell lines examined and is thought to be involved in centrosome amplification (14–16, 19). This study is the first to demonstrate a relationship between AIM-1 overexpression and chromosome missegregation because of increased mitotic H3 phosphorylation at Ser-10. There is a previous report of cytokinesis error attributable to AIM-1 overexpression (23), and some other effects of

AIM-1 overexpression have been noted as described above. A mutant H3 histone (S10E), which was engineered to mimic the constitutively phosphorylated form of H3 histone, induced chromosome lagging during mitosis when cells were transiently transfected with the mutant. However, no S10E-overexpressing cell clones were obtained after G418 selection of stable transfectants. The reason for this is not clear, but incorporation of S10E histone into chromatin may be toxic to cells. H3 histone is an essential component of chromatin, and the S10E histone is known to be constitutively incorporated into chromatin during interphase, as well as during mitosis. Thus, it is possible that the S10E histone interfered with normal chromatin function, once it was incorporated into the cells.

AIM-1 expression levels are regulated at both the mRNA and protein level, with maximal mRNA and protein levels occurring during the G_2-M phases (23). This cyclical pattern of regulation is conserved in cancer cells (21). This means that the effects of AIM-1 overexpression may be of critical importance during the G_2-M phases of the cell cycle. In the present study, increased H3 phosphorylation of Ser-10 attributable to AIM-1 overexpression was only observed in mitotic cells (Figs. 1*A* and 3*A*). CHE cells engineered to overexpress AIM-1 developed a malignant phenotype *in vivo* (Fig. 5). These tumor-bearing mice developed metastasis. Thus, mice injected with AIM-1-overexpressing cells that developed malignant phenotypes *in vivo* were more likely to dem-

onstrate metastasis. This observation agrees with our previous findings (22) in which we found a correlation between AIM-1 expression levels and Duke's grade in colorectal tumors. In the present experiment, both CIN and MIN colorectal tumor cell lines demonstrated AIM-1 overexpression and increased mitotic phosphorylation of histone H3 at Ser-10 (Fig. 6). Mitotic spindle checkpoint regulation is activated, even when there is only one lagging chromosome (46). AIM-1 overexpression induces chromosome lagging and, therefore, likely plays a role in spindle checkpoint activation. Indeed, in our recent experiments, AIM-1 overexpression was observed to activate the checkpoint pathway (47). Therefore, AIM-1 overexpression may be a driving force behind multistep carcinogenesis, occurring before CIN or MIN phenotypes are expressed. In addition, the fact that CHE cells with normal TP53 status did not yield any stable AIM-1-overexpressing clones in the present experiment suggests that AIM-1 overexpression may come into play after TP53 defects occur in the sequence of multistep carcinogenesis.

A Possible New Strategy for the Treatment of Cancer. It has been shown that *C. elegans* AIR-2, which is a functional homologue of AIM-1, acts with *C. elegans* Survivin-like protein BIR-1 to prevent mitotic apoptosis, and that Survivin is up-regulated in human cancer cells (48). AIM-1 is known to bind directly to both Survivin and INCENP (49). Because mitosis is the most susceptible phase of the cell cycle to various chemical and physical agents, the consistent observation of AIM-1 overexpression in cancer cells suggests that regulation of mitosis by AIM-1 signaling may be a possible target of cancer treatment. Identification of targets such as this should have a profound impact on cancer chemotherapy in the future.

ACKNOWLEDGMENTS

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