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AIM-1: a mammalian midbody-associated protein required for cytokinesis

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Mitosis is a highly coordinated process that assures the fidelity of chromosome segregation. Errors in this process result in aneuploidy which can lead to cell death or oncogenesis. In this paper we describe a putative mammalian protein kinase, AIM-1 (Aurora and Ipl1-like midbody-associated protein), related to *Drosophila* Aurora and *Saccharomyces cerevisiae* Ipl1, both of which are required for chromosome segregation. AIM-1 message and protein accumulate at G_2/M phase. The protein localizes at the equator of central spindles during late anaphase and at the midbody during telophase and cytokinesis. Overexpression of kinase-inactive AIM-1 disrupts cleavage furrow formation without affecting nuclear division. Furthermore, cytokinesis frequently fails, resulting in cell polyploidy and subsequent cell death. These results strongly suggest that AIM-1 is required for proper progression of cytokinesis in mammalian cells.
Keywords: Aurora/cleavage furrow/cytokinesis/Ipl1/midbody

Introduction

Cytokinesis occurs at the final stage of M phase and completes cell division. Mitosis is characterized by dramatic morphological changes, which occur in a strictly sequential order and include cytoskeletal disassembly, breakdown of the nuclear envelope, chromosome condensation, segregation and, finally, daughter cell separation. In the last stage of cytokinesis actin, myosin and other cytoplasmic molecules are redistributed into the cleavage furrow which forms midway between the segregated chromosomes in early anaphase (Cao and Wang, 1990). Actin–myosin interactions induce localized cortical contractions, which lead to an invagination of the plasma membrane in the cleavage furrow (Fishkind and Wang, 1995). Cytokinesis is accomplished by contraction of this actin ring and leads to separation of two daughter cells at the midbody. It remains to be shown how this last step is regulated.

Currently, the molecular basis of mitotic events is being studied extensively and some of these, such as entry into mitosis mediated by $p34^{cdc2}$ kinase and its associated cyclin subunits, are very well understood (Nurse, 1990). Protein phosphorylation has been suggested as an important regulatory mechanism for the elaborate processes of M phase events and many phosphoproteins are known to be associated with elements of the mitotic apparatus such as microtubule organizing centers, kinetochores and the spindle midbody (Davis *et al.*, 1983; Vandre *et al.*, 1984, 1991; Westendorf *et al.*, 1994; Drewes *et al.*, 1997). Various centrosomally localized protein kinases have been identified in vertebrate cells, including Cdc2 (Bailly *et al.*, 1989), Plk1 (Golsteyn *et al.*, 1995), Fyn (Ley *et al.*, 1994), Src (David-Pfeuty *et al.*, 1993) and A kinase (Nigg *et al.*, 1985). Some of these might function as regulators of microtuble organization and spindle formation. Whether these protein kinases are directly or indirectly regulated by Cdc2 kinase is at present only poorly understood. Furthermore, it is unknown whether mechanisms mediated by protein phosphorylation are also involved in regulation of cytokinesis.

Among these protein kinases the *Drosophila* serine/threonine protein kinase Aurora is known to be required for progression through M phase. Aurora was identified through recessive female sterile mutations, such that a mutant *aurora* mother would produce embryos in which centrosomes remained closely paired at inappropriate mitotic stages (Glover *et al.*, 1995). Loss of function of the protein kinase encoded by *aurora* leads to a failure of the centrosomes to separate and form a bipolar spindle. *Saccharomyces cerevisiae* Ipl1 kinase (Francisco *et al.*, 1994) is highly homologous to Aurora. Loss of Ipl1 function results in chromosome missegregation and cell death. Moreover, in these cells the intensities of DNA staining located near the opposite ends of many elongated spindles are uneven, indicating that unequal numbers of chromosomes segregate to the two poles. These data suggest that Aurora and Ipl1 play important roles in cell division and chromosome segregation. Several protein kinases related to Aurora and Ipl1 are known in other species, like *Xenopus* XLP46APK and XLP46BPK and mouse STK-1 (Niwa *et al.*, 1996). Aik/Ayk is a human homolog of Aurora and Ipl1-related kinases (Kimura *et al.*, 1997). The levels of Aik/Ayk mRNA and protein are induced during G_2/M and decrease rapidly after the end of mitosis. Aik/Ayk protein localizes at the spindle pole from prophase to anaphase. The data so far available for the Aurora and Ipl1 family members suggest that this molecule is evolutionally conserved and may be generally involved in M phase progression.

In this paper we report the isolation of a putative rat protein kinase, AIM-1, which is highly related to Aurora and Ipl1 and describe its possible involvement

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in cytokinesis. Immunolocalization experiments show that the AIM-1 protein is found exclusively in the equatorial plane (midzone) in late anaphase and in the midbody in telophase. Overexpression of a dominant negative type of AIM-1 affects formation of the cleavage furrow during late anaphase and leads to multinucleated cells. These data suggest that AIM-1 function is relatively specific for cytokinesis and point to a key role for AIM-1 in establishing the cleavage furrow as well as in the onset of cytokinesis. We discuss the role of AIM-1 in cytokinesis in relation to other gene products with similar functions.

Results

Identification and sequence of AIM-1

We screened a NRK-49F cDNA library using PCR for fragments encoding the VI–VII subdomain of serine/threonine-type protein kinases (Otsu *et al.*, 1993). The corresponding full-length clones of these 12 new cDNAs were isolated by screening the same library with these kinase cDNA fragments generated by PCR as probes. Subsequently, each of these cDNA inserts was subcloned into a *Saccharomyces cerevisiae* expression vector under control of the *GAL1/10* promoter and introduced into wild type *S.cerevisiae* cells on a multicopy vector. Galactose-induced overexpression of one gene among these 12 putative protein kinases was growth inhibiting and large budded cells with 2N DNA content were formed. Therefore, we further characterized this gene, which might be involved in mammalian G_2/M function. We named this new gene *AIM*-1 (Aurora and Ipl1-like midbody-associated protein).

The deduced amino acid sequence of AIM-1 consisted of 344 amino acid residues and gave a calculated molecular mass of 39.2 kDa. When we searched the latest available databases AIM-1 showed high homology to six serine/threonine-type protein kinases, STK-1, Aik/Ayk, XLP46APK, XLP46BPK, Aurora and Ipl1. In comparison with these molecules we discuss the AIM-1 protein structure as subdivided into the following three domains: (i) an 80 residue N-terminal header sequence; (ii) a catalytic domain containing the common protein kinase motifs (251 amino acid residues); (iii) a 13 residue C-terminal tail with a putative destruction box (Murray, 1995). The multiple alignment of AIM-1 with the six serine/threonine-type protein kinases is shown in Figure 1A. The kinase domain of AIM-1 shares 76, 73, 71, 60 and 46% identity with those of XLP46APK, XLP46BPK, Aik/Ayk, Aurora and Ipl1 respectively. The N-terminal header domain of AIM-1, however, shows little homology with Aik/Ayk, XLP46APK, XLP46BPK, Aurora and Ipl1. In contrast, human Aik (Kimura *et al.*, 1997)/mouse Ayk share a substantial degree of sequence similarity with *Xenopus* XLP46APK and XLP46BPK, not only in the catalytic domain, but also in the N-terminal domain (29 and 29%). As STK-1 (Niwa *et al.*, 1996), which was identified in mouse embryonic stem cells, was almost completely homologous to AIM-1 (93%) and the STK-1 protein had a molecular mass similar to that of AIM-1, AIM-1 appears to be a rat homolog of STK-1. Currently, except for Aurora, Ipl1 and Aik, the functions of these are not known. Amino acid sequence alignments of the kinase domains of Aurora and Ipl1-related kinases and other representative serine/threonine kinases were done using the Geneworks program and a hypothetical phylogenetic tree was drawn (Figure 1B). In this phylogenetic tree Aurora and Ipl1-related kinases were classified into a subfamily distinct from other kinases, including Cdk, Erk or Mek. This indicates that they are members of a unique 'Aurora and Ipl1-related kinase family' and that these kinases are conserved evolutionarily from yeasts to mammals. In mammals there are at least two genes, according to differences in the N-terminal header domain: AIM-1/STK1 and Aik/Ayk.

Tissue distribution

AIM-1 expression in various rat tissues was studied by Northern blotting with a cDNA fragment corresponding to the N-terminal header domain (Figure 2A). This analysis detected a 1.8 kb *AIM*-1-specific mRNA predominantly in testis, spleen and lung. Expression was exceptionally high in testis. Together with the 1.8 kb band, another faint band of 4.0 kb was also always identified. This 4.0 kb message may be from alternative splicing, since in rat NRK-49F cells this band showed the same cell cycle-specific oscillation pattern as the 1.8 kb band (data not shown).

Both AIM-1 message and protein are expressed in the G_2/M phase of the cell cycle

Since Aurora and Ipl1 are required for chromosome segregation in M phase, we investigated whether the AIM-1 expression pattern undergoes cell cycle-dependent oscillation. Poly(A)$^+$ RNA was prepared from synchronized NRK-49F cells at the indicated time points and analyzed by Northern blotting (Figure 2B). *AIM*-1 mRNA was most abundant 20 h after serum stimulation, indicating that the message was induced in late S phase, peaked near the G_2/M transition and decreased rapidly at the next G_1 phase. In addition, M phase arrest by colcemid treatment induced a marked accumulation of *AIM*-1 mRNA (data not shown). These data suggest that AIM-1 functions during M phase.

To study the roles of this protein during M phase an antibody against a synthetic peptide corresponding to the C-terminal 13 amino acids of AIM-1 was prepared (Figure 1). The results of Western blotting demonstrate the specificity of this antibody (Figure 2C). The affinity purified rabbit anti-AIM-1 antibody N12 detects an ~41 kDa protein (a slightly higher molecular weight than the predicted 39.2 kDa) in protein extracts from colcemid-treated NRK-49F cells (lane 2). AIM-1 protein was identified exclusively in the insoluble fractions. This protein co-migrated with *in vitro* translated AIM-1 products (lane 1). It is noteworthy that the expression level of AIM-1 in colcemid-arrested cells (lane 2) was ~5-fold higher than in asynchronous cells (lane 3).

We next studied AIM-1 protein level in NRK-49F cells during cell cycle progression (Figure 2D). Cells synchronized by serum starvation were harvested every 4 h after serum stimulation and extracts prepared. AIM-1 began to accumulate at the S/G_2 boundary, reached maximum level in G_2/M and decreased markedly in the next G_1 phase. Since serum-stimulated cells from G_0 may differ from continuously cycling cells, proteins

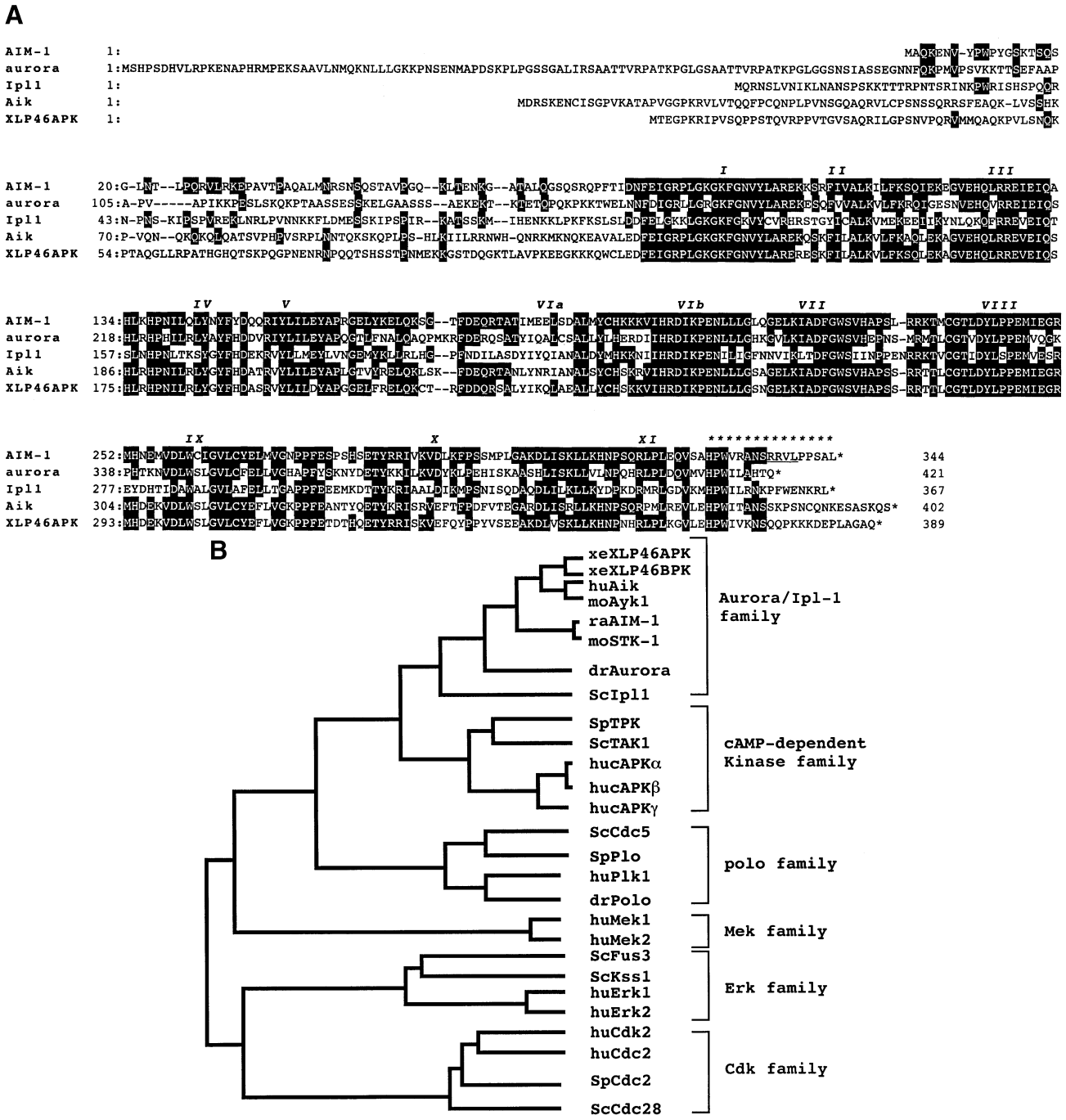


Fig. 1. Comparison of Aurora and Ipl1-related kinases from various organisms. (**A**) The predicted amino acid sequences of five Aurora and Ipl1-related kinases are aligned for maximum homology. Amino acid numbers are shown on the left. Roman numbers above the amino acid sequences show the kinase subdomains as described by Hanks *et al.* (1998) and Hanks and Hunter (1995). Residues identical to AIM-1 are shown in black. The amino acid sequence comprising AIM-1 subdomains VIb–VII is the region first identified by PCR. The amino acid sequence marked with asterisks was used to prepare the anti-peptide antibody. The underlined short stretch of the AIM-1 C-terminal amino acid sequence is the putative destruction box. (**B**) A hypothetical phylogenetic tree. The phylogenetic tree was drawn using the Geneworks program. xeXLP46APK, xeXLP46BPK, huAik, moAyk1, raAIM-1, moSTK, drAurora and ScIpl1 indicate the members of the Aurora and Ipl1-related kinase family from *Xenopus*, human, mouse, rat, *Drosophila* and *S.cerevisiae* respectively. Sp is *S.pombe*.

prepared from cells synchronized by double thymidine block and release were prepared. Similar results were obtained (data not shown). These data are consistent with the *AIM*-1 mRNA levels. Due to the high insolubility we have not yet succeeded in measuring the kinase activity of this protein. Since the C-terminus of AIM-1 contains the consensus motif for the putative destruction box (Figure 1), AIM-1 might undergo ubiquitin proteasome-dependent proteolysis, like other G_2/M phase regulators such as cyclin B (Glotzer *et al.*, 1991) and Cut2 (Funabiki *et al.*, 1996).

AIM-1 protein localizes to the midzone and the midbody

To examine the involvement of this molecule in the mitotic machinery during cell cycle progression, asynchronously

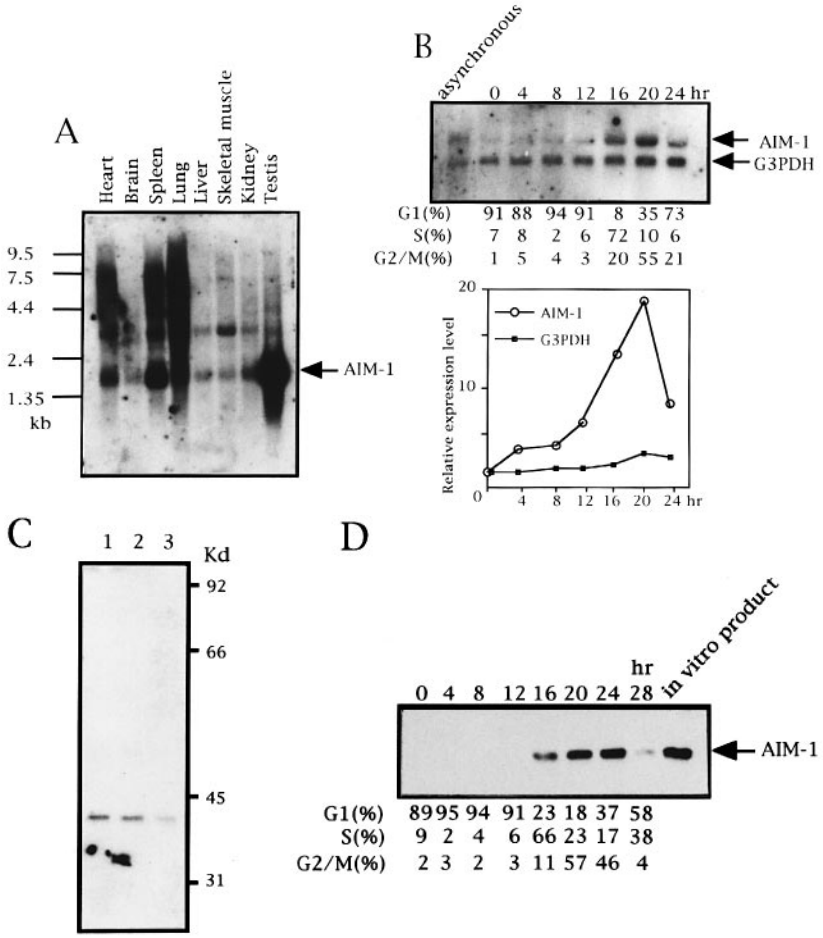


Fig. 2. AIM-1 is transcriptionally controlled during G_2/M phase. (**A**) Distribution of *AIM*-1 mRNA in various rat tissues. A membrane filter with mRNAs (2 μg) from various rat tissues (Clonetech) was probed with ^{32}P-labeled N-terminal *AIM*-1 DNA fragments. (**B**) *AIM*-1 mRNA is induced in late S and peaks near the G_2/M transition. Confluent rat NRK-49F cells were serum starved for 2 days. Cell cycle progression was started by replating the cells at a 1:3 ratio in serum-containing Dulbecco's modified Eagle's medium. At each cell cycle time point 2 μg poly(A)$^+$ RNA were analyzed with an *AIM*-1 cDNA probe. The control study was performed with a *G3PDH* gene probe (Nippon Gene). FACS analysis data are shown as the percentage for each time point. (**C**) Specificity of the anti-AIM-1 polyclonal antibody N12. Aliquots of the product (Promega) of an *in vitro* transcription/translation reaction (lane 1), total insoluble protein from NRK-49F cells arrested by colcemid treatment (lane 2) and asynchronous NRK-49F cells (lane 3) were separated by SDS–PAGE, blotted onto nitorocellulose and incubated with 5 μg/ml antibody N12. (**D**) AIM-1 protein is induced during G_2/M phase. Cell cycling was initiated under the same conditions as described in (B). Aliquots of the total insoluble protein corresponding to 2×10^5 cells at each time point were subjected to Western blot analysis with anti-AIM-1 antibody N12. As a control an aliquot of an *in vitro* transcription/translation reaction product of *AIM*-1 was used.

Table I. Effect of kinase-inactive AIM-1 on cell division

Plasmid	Cell lineage			
	NRK-49F (%)[a]	KD (%)	Mv1Lu (%)	
			+ doxycycline	– doxycycline
Vector	0.4 (1/232)[b]	0.6 (1/156)	0.4 (1/241)	0.7 (2/282)
AIM-1(WT)	7.8 (18/231)	4.8 (8/168)	0.9 (2/216)	3.0 (7/230)
AIM-1(K-R)	62.0 (161/260)	38.2 (68/178)	7.7 (20/261)	67.0 (156/233)

AIM-1(WT), AIM-1(K-R) or pEF/hyg I as a control vector was co-transfected with a reporter construct RSV–β-galactosidase into NRK-49F cells or KD cells. Cells were fixed 72 h after transfection and analyzed by microscopy. Expression of β-galactosidase was visualized as described in Materials and methods. The conditions used for Mv1Lu cells were as indicated in Figure 4B.
[a]The number shown is the percentage of cells with more than two nuclei. For NRK-49F or KD cells this was calculated based on the number of cells co-expressing β-galactosidase. For Mv1Lu cells this was calculated based on the total number of cells counted.
[b]Number of cells displaying the phenotype/total number of cells carrying plasmids.

growing NRK-49F cells were immunocytochemically studied with antibody N12. In interphase no obvious AIM-1 signal was detected (Figure 3A). However, spindle staining became visible as cells progressed through prophase, concomitant with concentration of AIM-1 in the pericentriolar material, while in metaphase AIM-1 became

dispersed in the cytoplasm (Figure 3B and C). In late anaphase AIM-1 began to be detectable as a distinct wide band extending across the midzone of the central spindle (Figure 3D). As the cells progressed to telophase and cytokinesis AIM-1 became more sharply concentrated at the midbody (Figure 3E and F).

Virtually identical results were obtained when we induced FLAG–AIM-1 fusion protein under tight control of a tetracycline-inducible system (Gossen and Bujard, 1992) in mink lung epithelial (Mv1Lu) cells (Figure 3G). Mv1Lu cells were transfected with both a construct with the tetracycline repressor fused to the activating domain of herpes simplex virus transcriptional activator VP16 and a construct containing the FLAG–*AIM*-1 cDNA under control of the tetracycline operon regulatory elements. This system allows expression of the desired protein only after depletion of doxycycline, a tetracycline analog, but not in the presence of the drug. Immunodetection of the induced FLAG–AIM-1 with anti-FLAG antibody demonstrated that FLAG–AIM-1 localized correctly to the midbody, suggesting that anti-AIM-1 antibody N12 specifically recognizes AIM-1 in cell immunostaining.

Since γ-tubulin is identified in the midbody and microtuble organizing centers (Julian *et al.*, 1993; Shu *et al.*, 1995), we also determined the subcellular localization of γ-tubulin in FLAG–AIM-1-expressing Mv1Lu cells. As shown in Figure 3G, FLAG–AIM-1 protein and γ-tubulin co-localized at the midbody. These data suggest that the appearance of AIM-1 protein in mitotic cells coincides precisely with the kinetics of protein expression shown by the Western blot data (Figure 2C) and that AIM-1 might regulate the process from anaphase to telophase/cytokinesis.

Induction of kinase-inactive AIM-1 causes abortive cytokinesis

In order to understand the role of AIM-1 during mitosis we examined the effects of kinase-inactive FLAG–AIM-1(K-R), in which the lysine residue of the ATP site at position 109 was replaced by arginine (Hanks *et al.*, 1988), to block function of the endogenous AIM-1 protein in a dominant negative manner. Cells carrying vector alone, wild-type FLAG–AIM-1(WT) or FLAG-AIM-1(K-R) were grown in the presence or absence of doxycycline for 24 h and then subjected to Western blotting with anti-FLAG antibody (Figure 4A). In the presence of 2 μg/ml doxycycline the basal levels of FLAG–AIM-1(WT) or FLAG–AIM-1(K-R) in exponentially growing cells were similar to the levels seen in cells transfected with vector alone. The expression of FLAG–AIM-1 was rapidly induced after doxycycline removal (Figure 4A). On Western blots these bands migrated slightly slower than those of the endogenous AIM-1 when detected with anti-AIM-1 antibody (data not shown). When FLAG–AIM-1(K-R) was induced for 72 h, ~67% of the cells failed to complete normal cytokinesis and they had more than two nuclei [FLAG AIM-1(WT): 3%] (Figure 4B). In addition, the size of these cells was much larger than that of normal cells (Figure 4B and D). In these abnormal cells, however, spindle functions, including chromosome disjunction and late anaphase spindle elongation, as well as nuclear division appeared unaffected (data not shown).

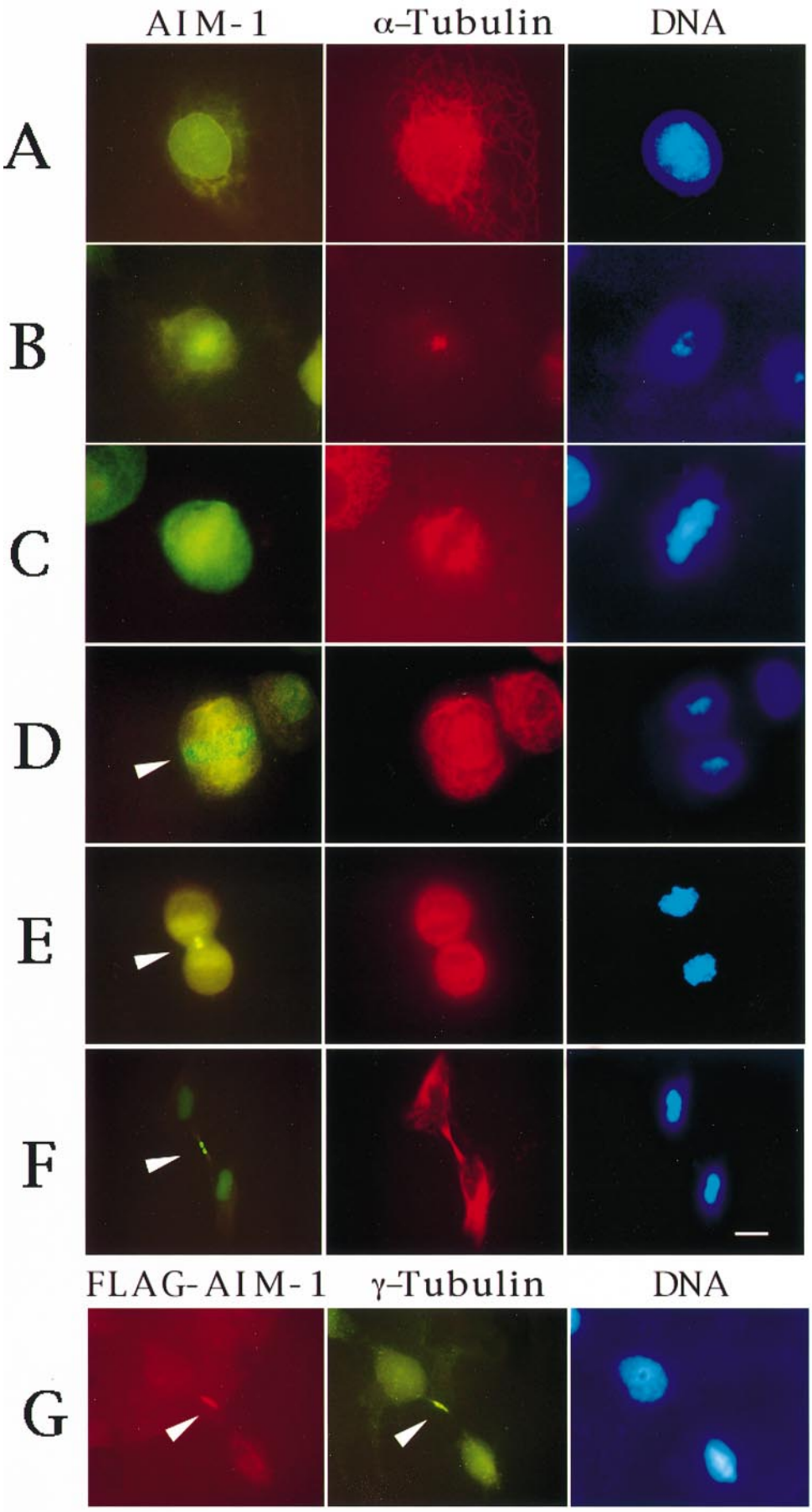
These results were confirmed by fluorescence-activated cell sorting (FACS) analysis (Figure 4C). When ethanol-fixed cells were analyzed, cell populations with 4N or 8N DNA content increased dramatically 72 h after induction of FLAG–AIM-1(K-R), coinciding with the appearance of binucleate or tetranucleate cells. Cells expressing FLAG–AIM-1(WT) or cells transfected with vector alone displayed normal cell cycle division patterns (Figure 4C). Additionally, we analyzed preparations of these cells by time-lapse video microscopy. The induction of AIM-1(K-R) caused the cells to arrest in or delay mitosis for a prolonged period (Y.Terada, unpublished results). Most of the cells failed to establish a cleavage furrow and eventually became binucleate. To test the generality of this observation, AIM-1(WT) or AIM-1(K-R) constructs were co-transfected transiently with RSV–β-galactsosidase (at a ratio of 10:1) into either rat NRK-49F cells or human diploid fibroblast KD cells. The β-galactosidase expression plasmid served as a positive internal control for successful expression of transfected DNA. Transfection of the vector alone did not affect cell cycle progression, but transfection of the AIM-1(K-R) construct resulted in accumulation of binucleate cells (Table I). However, transfection of AIM-1(WT) also caused abortive cytokinesis at a low level. These data suggest that expression of this protein at a proper level and/or timing is required to accomplish normal cytokinesis.

The nuclei of FLAG–AIM-1(K-R)-expressing cells could divide three to four times over a 4–5 day time period, resulting in >10 nuclei/cell (Figure 4D). Subsequently, however, these multinucleate cells did not divide further and failed to adhere to the plate. Indeed, when we tested cell growth in the presence or absence of doxycycline for 2 weeks to investigate the effects of AIM-1(K-R) on viability, the growth of Mv1Lu cells expressing AIM-1(K-R) but not AIM-1(WT) was inhibited (Figure 4E). These findings suggest that AIM-1 function is essential for cell viability.

Discussion

We have identified a putative rat protein kinase, AIM-1, related to *Drosophila* Aurora and *S.cerevisiae* Ipl1, both of which are known to be required for chromosome segregation. Cell cycle analysis shows that AIM-1 protein is induced periodically in G_2/M, suggesting that cell cycle oscillation of AIM-1 protein level is regulated predominantly in a transcriptional fashion around M phase. In addition, levels of AIM-1 protein decline sharply at the end of M phase. This might be due to degradation of the protein through the presumptive ubiquitination destruction box in the C-terminal tail of AIM-1. Immunolocalization studies demonstrate that AIM-1 protein undergoes rapid redistribution during M phase. AIM-1 localizes at the spindle poles in prophase, redistributes to the spindle midzone in late anaphase and finally becomes even more sharply concentrated at the midbody region as late anaphase progresses into telophase.

Overexpression of putative kinase-inactive AIM-1 in a tetracycline-inducible system leads to a failure of cytokinesis, which is consistent with the markedly increased numbers of multinucleate cells with 4N or 8N DNA content. To explain this observation we suggest that kinase-negative AIM-1 competes with endogenous AIM-1 for


AIM-1
α-Tubulin
DNA
A
B
C
D
E
F
FLAG-AIM-1
γ-Tubulin
DNA
G

the midzone and midbody binding sites, thus reducing the amount of functional AIM-1 at the midzone and midbody. These lines of evidence strongly indicate that AIM-1 is required for the onset of cytokinesis. This hypothesis is also supported by the finding that AIM-1 localizes at the midzone and the midbody. Moreover, immunofluorescent staining with anti-α-tubulin, DNA staining and FACS analysis of multinucleate cells revealed that normal bipolar spindles are formed and normal nuclear division occurs in cells depleted of AIM-1 function. These data suggest that AIM-1 participates specifically in regulation of cytokinesis. Nevertheless, since the centrosome in prophase is immunologically stained with AIM-1 antibody, AIM-1 might be involved in other functions during M phase.

These conclusions are, however, in contrast to the fact that *aurora* mutations show abnormal mitotic spindles due to failure of the centrosomes to separate and form bipolar spindles (Glover *et al.*, 1995). Conditional expression of a kinase-negative AIM-1 did not affect formation of normal bipolar spindles and chromosome segregation. This suggests that AIM-1 might simply be related to Aurora rather than a true functional homolog. Recently Aik (Aurora/Ipl1-related protein kinase)/Ayk was identified as a candidate for a mammalian homolog with Aurora function (Kimura *et al.*, 1997). In addition, Aik appears to play an important role for centrosome separation (Kimura *et al.*, personal communication). These data suggest that Aik/Ayk is a true functional homolog of Aurora and that

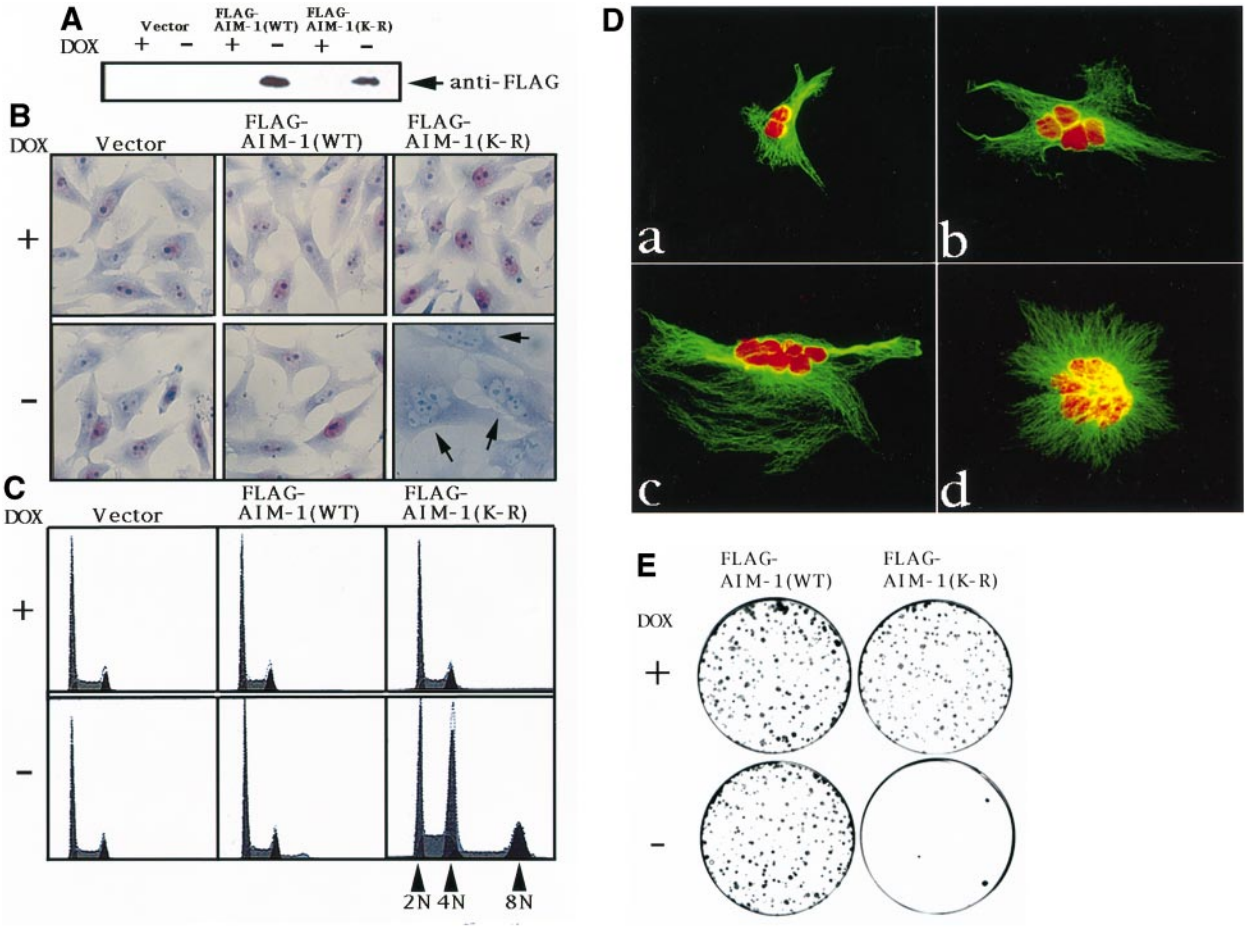


Fig. 4. Induction of kinase-inactive AIM-1 abrogates cytokinesis. (**A**) Induction of wild-type FLAG–AIM-1(WT) or kinase-inactive FLAG–AIM-1(K-R) in Mv1Lu cells. The cells were cultured for 24 h after doxycycline (DOX) removal. Lysates were immunobloted with anti-FLAG mAb (M2). (**B**) Overexpression of kinase-inactive AIM-1 leads to multinucleate cells. Asynchronous cells were transfected with vector alone, FLAG–*AIM*-1(WT) or FLAG–*AIM*-1(K-R), and grown with or without doxycycline for 72 h. Cells were subsequently stained with Giemsa solution. Cells expressing FLAG–AIM-1(K-R) had more than two nuclei, which are the clear areas (arrow) surrounding the dark dots of nucleoli. (**C**) FACS analysis demonstrates the increased DNA content after expression of kinase-inactive AIM-1. Cells were harvested as described in (B). For FACS analysis cells were fixed with ethanol and stained with propidium iodide. (**D**) Multinucleate cells induced by AIM-1(K-R) expression. Double staining for α-tubulin (green) and DNA (red; propidium iodide) was displayed by superimposing the respective images of Mv1Lu cells expressing FLAG–AIM-1(K-R) as described in (B). AIM-1(K-R) expression induced the appearance of abnormal cells each containing two (a), four (b), eight (c) or more than 10 nuclei (d) after doxycycline removal. α-tubulin was detected with anti-α-tubulin mAb and FITC-conjugated goat anti-mouse IgG (Cappel) and the signals observed by confocal laser microscopy (Fluoview, Olympus). (**E**) AIM-1 function is essential for cell viability. Mv1Lu cells transfected with FLAG–*AIM*-1(WT) or FLAG–*AIM*-1(K-R) were grown with or without doxycycline for 2 weeks and stained with Giemsa solution.

Fig. 3. AIM-1 localizes to the midzone and the midbody. NRK-49F cells were stained with anti-AIM-1 polyclonal antibody (left column), anti-α-tubulin mAb (middle column) and Hoechst 33258 for DNA staining (right column): (**A**) interphase; (**B**) prophase; (**C**) metaphase; (**D**) late anaphase; (**E**) telophase; (**F**) cytokinesis. (**G**) Co-localization of FLAG–AIM-1 with γ-tubulin during cytokinesis. Mv1Lu cells carrying pUHD 10-3/FLAG–AIM-1(WT) were grown without doxycycline for 24 h and stained with anti-FLAG mAb M2, rabbit anti-γ-tubulin antibody and Hoechst 33258. Scale bar 10 μm.

it plays a major role in mammalian centrosome separation. Aik mRNA and protein are low in G_1/S, accumulate during G_2/M and decrease rapidly after mitosis. Its protein kinase activity is also enhanced in mitosis, as inferred by phosphorylation of exogenous casein. These Aik characteristics are very similar to those of AIM-1, but there is a difference between these two kinases. AIM-1 localizes at the midzone during anaphase and at the midbody during telophase, but Aik does not. This difference in localization could be explained by the different N-terminal header domain sequences between AIM-1 and Aik. The characteristic N-terminal header domain of AIM-1 is most likely responsible for association of AIM-1 protein with the midbody structure. This indicates that there are at least two genes in the mammalian Aurora and Ipl1-related kinase family: Aik is involved in establishment of the spindle pole body and AIM-1 plays a crucial role in regulating cytokinesis.

Spindle functions such as centrosome separation and initiation of cytokinesis are reported to be regulated by kinesin-like proteins (KLPs; Goldstein, 1991; Moore and Endow, 1996). In *Schizosaccharomyces pombe* the *cut7*$^+$ gene, which encodes a spindle-associated KLP, is required for spindle pole body separation (Hagan and Yanagida, 1990). In a *cut7* mutant microtubles nucleated from spindle pole bodies fail to interdigitize with one another. *Drosophila* KLP-61F, which is a member of the bimC/Cut7$^+$/Eg5 family (Enos and Morris, 1990; Hagan and Yanagida, 1990, 1992; Le Guellec *et al.*, 1991), is reported to be required for centrosome segregation (Heck *et al.*, 1993). The phenotypes of the *KLP-61F* and *aurora* mutations show strikingly similar abnormalities of the centrosomes (Glover *et al.*, 1995), thus suggesting that they might play regulatory roles in a common process and that the Aurora kinase might be involved in KLP-61F function to regulate kinesin motor activity. Recently KLP-3A, another member of the *Drosophila* KLP family, is reported to be a component of the midbody and to be required for central spindle assembly as well as initiation of cytokinesis during meiotic division in males (Williams *et al.*, 1995). The phenotype of *KLP-3A* mutations, which leads to the appearance of spermatids containing multiple nuclei, resembles the AIM-1 loss-of-function phenotype and KLP-3A and AIM-1 show similar intracellular distributions. Therefore, AIM-1 might regulate a mammalian KLP-3A homolog in cytokinesis, whereas Aik might control a member of the KLP-61F/ bimC/Cut7$^+$/Eg5 family. A number of proteins have been found to relocate from various parts of the spindle to the midzone during telophase, including Inner Centromere Protein (INCENP; Cooke *et al.*, 1987) and Telophase Disc 60 (TD60: Andreassen *et al.*, 1991). The behavior of these proteins suggests that they may contribute to the signaling of cytokinesis (Wheatley and Wang, 1996; Eckley *et al.*, 1997). Whether AIM-1 has close functional relationships with these proteins to complete cytokinesis remains to be shown.

In *Drosophila* another protein kinase, Polo, has been described as being involved in M phase progression (Fenton and Glover, 1993). Mutations in *polo* result in a high frequency of abnormal mitoses and aberrant chromosome segregation in larval neuroblasts. These cells frequently display hypercondensed chromosomes, monopolar spindles and disorganized spindle poles, leading to polyploidy. The mammalian M phase-specific protein kinase Plk1, which is related to Polo, is cell cycle regulated and peaks during late G_2 phase. Immunolocalization studies show that Plk1 localizes at the spindle pole during prophase and metaphase, redistributes to the equatorial regions of the spindle during anaphase and concentrates within the midbody (Golsteyn *et al.*, 1995; Lee *et al.*, 1995). These features of Polo and Plk1 are similar to those of AIM-1, though neither kinase is structurally homologous to AIM-1. Plo1, a *S.pombe* functional homolog of Polo, is another important regulatory molecule involved in cytokinesis (Ohkura *et al.*, 1995). Loss of Plo1 affects both actin ring formation and deposition of septal material and overexpression of Plo1 can trigger septation from any point in the cell cycle. These results suggest that fission yeast Plo1 kinase plays a dual role in both establishment of a bipolar spindle and in septum formation. Whether mammalian Plk1 performs equivalent functions in cytokinesis is currently unclear. Interestingly, Plk1 localizes not only at the centrosome but also at the midzone and the midbody, as does AIM-1. This suggests that mammalian Plk1 might have the same dual functions as fission yeast Plo1. If Plk1 is involved in control of mammalian cytokinesis, there might be interactions between Plo1 and AIM-1 during cytokinesis.

Nuclear division and cytokinesis are normally coordinated in the cell cycle and cytokinesis occurs exactly after completion of nuclear division. This implies the existence of a checkpoint control that suppresses the onset of cytokinesis before completion of nuclear division. Since in cells without normal AIM-1 function cytokinesis is dissociated from nuclear division, it is likely that AIM-1 coordinates cytokinesis with the mitotic events of the cell cycle. If this is the case, several questions would arise as to whether AIM-1 kinase is activated normally just after completion of nuclear division and what mechanisms exist to perform cytokinesis in the signaling system transduced by this kinase. To solve these questions we need to understand better how the actin ring is formed and how its contraction in cytokinesis is regulated. However, the kinase activity of AIM-1 has not yet been successfully determined due to its insolubility.

In summary, our data provide the first evidence that the novel serine/threonine-type kinase AIM-1 is essential for cytokinesis. Our results suggest a role for this protein in coordinating cytokinesis with the mitotic events of the cell cycle. However, at present little is known about the molecular events upstream or downstream of AIM-1 at the last stage of cell division. Further analyses of the signaling cascade which AIM-1 propagates could help clarify how the onset of cytokinesis is regulated relative to completion of nuclear division.

Materials and methods

AIM-1 cDNAs and expression plasmids

Full-length *AIM*-1 cDNAs were isolated from a rat NRK-49F cDNA library (Terada *et al.*, 1995). A kinase-inactive (K-R) mutant allele of *AIM*-1 was generated by site-directed mutagenesis using PCR. In AIM-1(K-R) the critical lysine (K) at position 109 in the putative ATP binding site was mutated to arginine (R). An N-terminal FLAG tag was added by PCR to full-length *AIM*-1(WT) and *AIM*-1(K-R) and they were subcloned into vectors pUHD 10-3 (Gossen and Bujard, 1992) and pEF/

hyg I (with the EF1α promoter and hygromycin-resistance marker; Terada *et al.*, 1995) respectively. These plasmids were transfected into Mv1Lu cells (ATCC) carrying pUHD 15-1 (Gossen and Bujard, 1992) either with the pEF/Hyg I vector (as a selection marker) for pUHD 10-3 constructs or alone for pEF/Hyg I constructs, using Lipofectamine according to the manufacturer's recommendations (Gibco-BRL). Clones were selected with hygromycin (0.2 mg/ml; Calbiochem) in the presence of doxycycline (2 μg/ml; Sigma) and those in which FLAG–AIM-1(WT) or FLAG–AIM-1(K-R) was induced 24 h after doxycycline depletion were chosen for further analysis. The expression of FLAG-tagged AIM-1 proteins was detected by Western blotting with anti-FLAG mAb (M2; Kodak). A clone carrying the empty vector alone was used as a control.

Antibodies

For preparation of a polyclonal antibody against AIM-1 (N12) a peptide located at the C-terminus of AIM-1 (HPWVRANSRRVLPPSAL) was synthesized, coupled to keyhole limpet hemocyanin and injected into rabbits. To obtain the anti-AIM-1 antibody, a glutathione *S*-transferase (GST)–AIM-1 C-terminus fusion protein was produced in bacteria. The antibody was purified from whole serum using GST–AIM-1 C-terminus on an affinity column.

Cell extract preparation

Cells were extracted with RIPA buffer (10 mM Tris–HCl, pH 7.5, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl flouride, 4 mg/ml leupeptin), followed by centrifugation at 100 000 *g* for 1 h. The pellets were suspended in SDS–PAGE sample buffer, boiled and sonicated. The supernatant after centrifugation at 10 000 *g* was treated with SpinBind (FMC) to deplete contaminating DNA fragments.

Cell immunostaining

Cells grown on Labtek chamber slides (Nunc) were pretreated with a microtubule stabilizing buffer (MSB; 80 mM potassium PIPES, pH 6.8, 5 mM EGTA, 1 mM $MgCl_2$) containing 0.5% Triton X-100 and then fixed with methanol (10 min at –20°C) as described by Boleti *et al.* (1996). Fixed cells were washed with a solution of 0.1 M PIPES, pH 7.2, 1 mM $MgSO_4$, 1 mM EGTA, 1.83% L-lysine, 1% BSA and 0.1% sodium azide and were subsequently incubated for 1 h with a monoclonal mouse anti-α-tubulin mAb 7-5168 (1 μg/ml) (Sigma) and affinity purified rabbit AIM-1 antibody (N12) (5 μg/ml) (for Figure 3A–F) or mouse anti-FLAG mAb (M2) (1 μg/ml) and rabbit anti-γ-tubulin antibody (at 1:600 dilution) (Masuda and Shibata, 1996) (for Figure 3G). This was followed by incubation with FITC-conjugated goat anti-rabbit IgG antibody (Cappel) and rhodamine-conjugated goat anti-mouse IgG antibody (Cappel). The stained cells were observed with conventional fluorescent (Nikon) or confocal laser microscopes (Fluoview, Olympus).

β-galactosidase cytochemistry

To visualize expression of the reporter enzyme β-galactosidase transfected cells on glass coverslips were fixed in 1.25% glutalaldehyde in phosphate-buffered saline (PBS) for 5 min at room temperature and washed twice with PBS. Cells were then stained in X-gal buffer (50 mM Tris–HCl, pH 7.5, 2.5 mM potassium ferrocyanide, 2.5 mM potassium ferricyanide, 15 mM NaCl, 1 mM $MgCl_2$, 0.5 mg/ml X-gal) for 2–4 h.

Nucleotide sequence accession number

The DDBJ/EMBL/GenBank accession No. for the AIM-1 sequence reported in this paper is D89731.

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