sion (*3*, *4*, *9*, *11*, *23*, *24*). APC and GSK3β have been implicated in the control of β-catenin stability. We demonstrate here that conductin interacts with β-catenin, APC, and GSK3β. Conductin overexpression directs β-catenin to the degradation pathway without functional APC. Mutants of conductin act antagonistically in this pathway. Moreover, APC fragments that interfere with conductin-APC interaction inhibit degradation of β-catenin. Conductin, therefore, is positioned downstream of APC in this regulatory pathway. The related protein axin, which suppresses axis formation in mice (*15*), might function similarly.

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12. Yeast two-hybrid screens were performed with the region of β-catenin encoding the 13 arm repeats (*1*) or with the RGS domain of conductin (amino acids 78 to 200) in a DNA binding domain vector and a cDNA library from 10.5-day mouse embryos in a VP16 activation domain vector (*6*). Full-length conductin was isolated from λgt10 mouse embryo cDNA libraries (Stratagene). Deletion mutants of conductin and β-catenin were made by restriction digests or polymerase chain reaction and tested for interaction in a quantitative β-galactosidase assay. Sequence comparisons were made with BLAST and ClustalW (1.7) programs [S. F. Altschul, W. Gish, W. Miller, E. M. Myers, D. J. Lipman, *J. Mol. Biol.* **215**, 403 (1990)].
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16. Mutations were introduced [K. M. Weidner *et al.*, *Nature* **384**, 173 (1996)] into the APC fragment #4, and interactions with the RGS domain of conductin were measured in yeast two-hybrid assays. The SAMP repeat was mutated from SAMP to AALP.
17. Transfection of mammalian cells, immunoprecipitations, and protein immunoblots were performed as described (*1*) with antibody to β-catenin (anti–β-catenin), anti-GSK3β, anti–myc tag (9E10), and anti-flag (Kodak). Antibodies to conductin were prepared in rabbits by immunization with a recombinant fragment of conductin containing amino acids 7 to 396. The antibody was purified by affinity chromatography and shown to be specific for conductin by immunoprecipitation and protein immunoblot analysis. Anti-GSK3β was purchased from StressGen (Victoria, Canada). Immunofluorescence staining was performed on cells fixed with 2% formaldehyde and permeabilized with Triton X-100 (*1*).
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21. The degree of ventralization of *Xenopus* embryos was dependent on the concentration of the injected conductin mRNA. We noticed a decrease in β-catenin concentrations in the injected embryos. Axis formation was rescued by co-injection of mRNA that encodes LEF-1 but not of a LEF-1 deletion mutant that lacks the NH_2-terminal β-catenin binding domain. Axis formation was also rescued by co-injection of mRNAs encoding β-catenin or siamois but not Xwnt-8. The mRNA for *Xenopus* injection was produced by in vitro transcription with the pSP64T3 vector and SP6 RNA polymerase [M. Kühl, S. Finnemann, O. Binder, D. Wedlich, *Mech. Dev.* **54**, 71 (1996)] and was injected into dorsal blastomeres of 4-cell-stage embryos. Control injections of prepro-lactin mRNA resulted in no morphological changes.
22. A 4.7-kb conductin mRNA was found in mouse embryos by Northern (RNA) blot analysis at all developmental stages tested (E7 through E17) and in many adult tissues; highest expression levels in the adult are observed in brain, lung, and liver. In situ hybridization of E11 and E12 mouse embryos revealed distinct patterns of conductin expression, for example, in the dorsal neural tube, distinct regions of the brain, the mesenchyme below the epidermis, lung mesenchyme, and kidney epithelia. Multiple-tissue Northern blots (Clontech) and in situ hybridization of mouse embryos were carried out as described [F. D. Bladt, D. Riethmacher, D. S. Isenmann, A. Aguzzi, C. Birchmeier, *Nature* **376**, 768 (1996)].
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25. Sequences related to the SAMP repeat are found in APC of *Caenorhabditis elegans* [C. E. Rocheleau *et al.*, *Cell* **90**, 707 (1997)]. The published APC sequence of *Drosophila* does not contain SAMP repeats [S. Hayashi *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 242 (1997)].
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27. We thank C. Birchmeier for helpful suggestions to the manuscript, S. Britsch and S. Dietrich for help in the analysis of mouse embryos, V. Brinkmann for help with confocal microscopy, and K. Feller and B. Bradtke for technical assistance. The Xwnt-8 construct was a gift from R. Moon, University of Washington, Seattle, and the siamois construct a gift from P. Lemaire, Institute de Biologie du Developpement de Marseille, France.

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Activation of the Protein Kinase p38 in the Spindle Assembly Checkpoint and Mitotic Arrest

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The mitogen-activated protein kinase (MAPK) superfamily comprises classical MAPK (also called ERK), c-Jun amino-terminal or stress-activated protein kinase (JNK or SAPK), and p38. Although MAPK is essential for meiotic processes in *Xenopus* oocytes and the spindle assembly checkpoint in *Xenopus* egg extracts, the role of members of the MAPK superfamily in M phase or the spindle assembly checkpoint during somatic cell cycles has not been elucidated. The kinase p38, but not MAPK or JNK, was activated in mammalian cultured cells when the cells were arrested in M phase by disruption of the spindle with nocodazole. Addition of activated recombinant p38 to *Xenopus* cell-free extracts caused arrest of the extracts in M phase, and injection of activated p38 into cleaving embryos induced mitotic arrest. Treatment of NIH 3T3 cells with a specific inhibitor of p38 suppressed activation of the checkpoint by nocodazole. Thus, p38 functions as a component of the spindle assembly checkpoint in somatic cell cycles.

MAPKs are serine-threonine kinases that are activated by various mitogens that induce transition from the quiescent state into the cell cycle division (*1*). MAPK is also activated during meiotic maturation in *Xenopus* oocytes and has an essential role in both the transition from G_2 to M phase of meiosis and metaphase arrest of mature oocytes (*2*–*4*). Although these results suggested the possible role of MAPK/ERK in the mitotic M phase also, little or no activation of MAPK is detected during M phase of somatic cell cycles. In an in vitro cell cycle system derived from *Xenopus* egg extracts, MAPK is required for the spindle assembly checkpoint mechanism (*5*, *6*), a mechanism conserved evolutionarily and essential for accurate transmission of genetic information to the daughter cells (*7*). Although a requirement for MAPK in the spindle assembly checkpoint in somatic cells has also been suggested because injection of MAPK phosphatase (XCL100) overcomes the checkpoint (*8*), activation of MAPK in cells arrested in M phase as a result of spindle assembly defects has not been observed. However, one or more MAPK-related molecules appears to be activated in the nocodazole-treated cells arrested in M phase (*9*). We therefore tested whether JNK or p38 (also known as MPK2, CSBP, or HOG1) might participate in the spindle assembly checkpoint of somatic cell cycles.

Lysates of nocodazole-arrested mitotic NIH 3T3 cells released from flasks by shak-

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ing contained kinase activity toward histone H1, a measure of maturation-promoting factor (MPF), confirming that the released cells were in M phase (Fig. 1A). Immune-complex kinase assays with antibody to p38 (anti-p38) revealed that the activity of p38 from mitotic cell lysates was three to four times greater than that from interphase cells, although the precipitates contained nearly equal amounts of p38 (Fig. 1B). These results indicate that p38 is activated in nocodazole-arrested mitotic cells. The activation of p38 in nocodazole-treated mitotic cells was comparable to that in cells treated with arsenite or anisomycin, which are activators of p38 (Fig. 1C). By contrast, JNK was not activated in nocodazole-arrested cells, although it was activated in cells treated with arsenite or anisomycin. MAPK was also not activated in nocodazole-arrested cells. Activation of p38 was also observed in nocodazole-arrested HeLa cells (Fig. 1D).

In nocodazole-treated cells, removal of nocodazole releases cells from mitotic arrest, and the cells progress from M to G_1 phase. We investigated the activity of p38 in such cells. The inactivation of histone H1 kinase activity (Fig. 2A) and microscopic observation (*10*) confirmed that the cells exited from M phase. Immune-complex kinase assays showed that p38 was inactivated before MPF after the release from the nocodazole arrest (Fig. 2A). This result is consistent with the idea that activated p38 functions to maintain the activity of MPF.

The kinase p38 is activated in response to various kinds of stresses (*11*). To determine whether nocodazole-induced activation of p38 occurred specifically in M phase cells as a result of the spindle assembly defect or nonspecifically irrespective of the cell cycle, we treated cells in various phases of the cell cycle with nocodazole and assayed the activity of p38. Cells were synchronized in G_0 phase by culture without serum and then induced to enter the cell cycle by addition of serum. Nocodazole treatment for 4 hours did not cause activation of p38 when most cells were in G_1 or S phase, but did stimulate the activity of p38 when most of the cells were in M phase (Fig. 2B). To examine whether p38 is activated in normal M phase without nocodazole, we synchronized HeLa cells at the G_1-S phase boundary by a double thymidine block and released them into the cell cycle. In the absence of nocodazole, most cells proceeded through M phase about 9 hours after release and then progressed to interphase (Fig. 2C). During this period, activa-

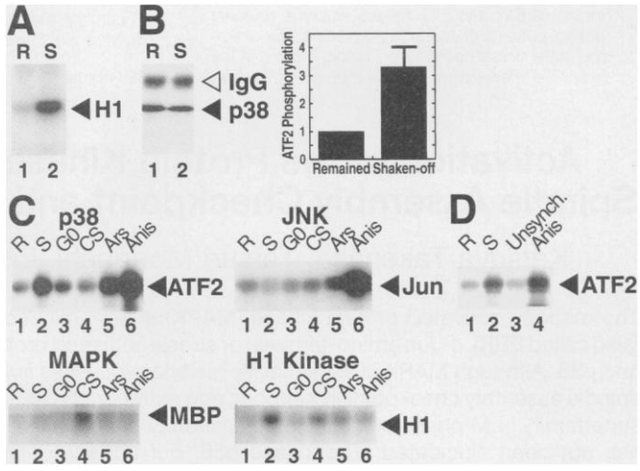

Fig. 1. Activation of p38 in nocodazole-arrested cells. (A and B) Lysates were obtained from nocodazole-arrested mitotic NIH 3T3 cells that were prepared by mechanical shake-off from flasks (S, lane 2) or from cells that remained on the flasks (R, lane 1) (*18*). (**A**) MPF activities in lysates were measured as histone H1 kinase activities (*19*). (**B**) Proteins were immunoprecipitated with anti-p38, and p38 in the precipitates was detected by immunoblotting (left) and assayed for kinase activity toward activating transcription factor 2 (ATF2) (right) (*20*). The result of the kinase assay represents the average of three independent experiments and is shown as a fold increase. IgG, immunoglobulin G. (**C**) Kinase activities of the members of the MAPK superfamily were measured by immune-complex kinase assays with ATF2, Jun, myelin basic protein (MBP), or histone H1 as substrate (*21*). NIH 3T3 cells were deprived of serum (lane 3) and stimulated by addition of calf serum (10%) for 5 min (CS, lane 4). Growing cells were stimulated by 50 μM arsenite for 15 min (Ars, lane 5) or by anisomycin (1 μg/ml) for 30 min (Anis, lane 6). (**D**) Lysates were obtained from HeLa cells as described for NIH 3T3 cells and assayed for p38 activity.

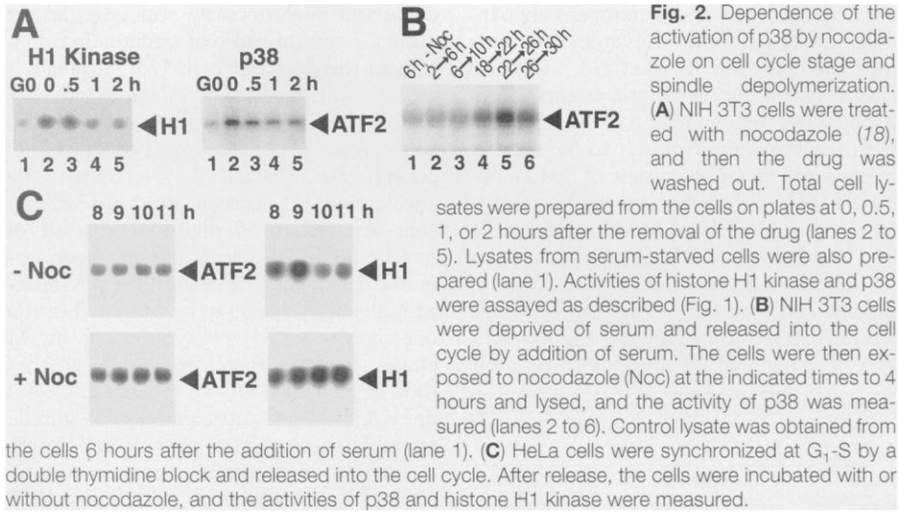

Fig. 2. Dependence of the activation of p38 by nocodazole on cell cycle stage and spindle depolymerization. (**A**) NIH 3T3 cells were treated with nocodazole (*18*), and then the drug was washed out. Total cell lysates were prepared from the cells on plates at 0, 0.5, 1, or 2 hours after the removal of the drug (lanes 2 to 5). Lysates from serum-starved cells were also prepared (lane 1). Activities of histone H1 kinase and p38 were assayed as described (Fig. 1). (**B**) NIH 3T3 cells were deprived of serum and released into the cell cycle by addition of serum. The cells were then exposed to nocodazole (Noc) at the indicated times to 4 hours and lysed, and the activity of p38 was measured (lanes 2 to 6). Control lysate was obtained from the cells 6 hours after the addition of serum (lane 1). (**C**) HeLa cells were synchronized at G_1-S by a double thymidine block and released into the cell cycle. After release, the cells were incubated with or without nocodazole, and the activities of p38 and histone H1 kinase were measured.

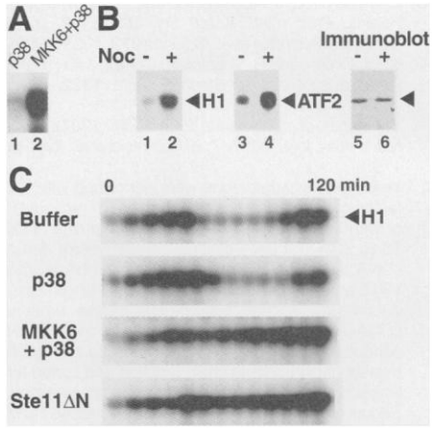

Fig. 3. Mitotic arrest induced by activated p38 in cell cycle extracts. (**A**) Recombinant p38 alone (lane 1) or a mixture of recombinant MKK6 and recombinant p38 (lane 2) was incubated with ATP, and then assayed for phosphorylation of ATF2 (*17*). MKK6 alone showed no kinase activity toward ATF2. (**B**) Activation of exogenous GST-p38 in the extracts treated with nocodazole. *Xenopus* egg extracts were incubated for 80 min at room temperature with recombinant GST-p38, recombinant MKK6, and 9000 sperm nuclei per microliter in the presence or absence of nocodazole (Noc, 10 μg/ml) (*22*). MPF activities were measured as histone H1 kinase activities (lanes 1 and 2). GST-p38 was immunoprecipitated with anti-GST and assayed for kinase activity toward ATF2 (lanes 3 and 4). Immunoprecipitates were immunoblotted with an anti-GST (lanes 5 and 6). (**C**) *Xenopus* egg extracts were incubated with buffer, p38, MKK6+p38, or Ste11ΔN (*23*). Samples were withdrawn at 10-min intervals and assayed for histone H1 kinase activity.

tion of p38 was not observed, whereas in cells treated with nocodazole, both MPF and p38 were activated. Thus, nocodazole treatment appears to activate p38 by disrupting spindle formation in M phase cells.

Our results raised the possibility that p38 functions in the spindle assembly checkpoint. The spindle assembly checkpoint is the mechanism that prevents cells from initiating anaphase and leaving mitosis until the spindle has been fully assembled. We therefore examined whether the activated p38 could cause arrest of the cell cycle in M phase. Although purified recombinant p38 expressed in *Escherichia coli* exhibited only weak kinase activity (Fig. 3A), after incubation with recombinant histidine-tagged MAPK kinase 6 (MKK6), a specific activator of p38, it became strongly activated (Fig. 3A). In the cell cycle of *Xenopus* egg extracts, MAPK is required for the spindle assembly checkpoint (*5*, *6*), and activation of MAPK alone by Ste11ΔN, a constitutively active MAPK kinase kinase (MAPKK-K), is sufficient to induce mitotic arrest (*6*) (Fig. 3C). In this system, the amounts of endogenous p38 (MPK2) and XMEK3 (a *Xenopus* homolog of MKK6) are low (*12*). When recombinant p38 and MKK6 were added to the extracts, the added p38 was activated during mitotic arrest induced by nocodazole (Fig. 3B). Thus, although MAPK is responsible for the checkpoint in the extracts (probably because of the much larger amount of MAPK than p38), the signal produced by the spindle depolymerization could be transmitted through p38 as well. We examined whether active p38 could arrest cell cycle extracts in M phase in the absence of nocodazole. Upon incubation at room temperature, periodic activation of histone H1 kinase activity occurred in the extracts with no additives (Fig. 3C). Addition of purified recombinant p38 that had not been activated did not affect the periodic activation and deactivation of MPF. In contrast, when recombinant p38 that had been activated with MKK6 was added to the extracts, high MPF activity was maintained after the first M phase (Fig. 3C). Thus, activated p38 can arrest the in vitro cell cycle in M phase.

To examine whether p38 can also induce mitotic arrest in the cell cycle in vivo, we microinjected activated p38 into one blastomere of a two-cell *Xenopus* embryo (*13*). This resulted in cleavage arrest usually at the two- or four-cell stage (Fig. 4A). About 37% of the embryos were arrested in the two-cell stage, about 39% in the four-cell stage, and about 5% in the later stages. Injection of buffer alone induced no cleavage arrest. The embryos injected with the activated p38 had higher histone H1 kinase activity than those injected with buffer (Fig. 4B), suggesting that the cleavage arrest was a mitotic arrest. Because endogenous MAPK was not activated by treatment of embryos or extracts with activated p38 plus MKK6 (*10*), we can exclude the possibility that the added MKK6 might act by way of MAPK. These results thus indicate that p38, if activated, can arrest the cell cycle in M phase (*14*).

To examine whether inhibition of p38 could cause loss of the checkpoint function, we used a specific inhibitor of p38, SB203580 (*15*). We treated synchronized NIH3T3 cells with or without SB203580 and then exposed the cells to nocodazole. Activation of MPF by nocodazole was suppressed in cells treated with SB203580 (Fig. 5A). Moreover, when SB203580 was added to nocodazole-arrested cells, the activity of MPF decreased with time (Fig. 5B). Thus, the spindle assembly checkpoint function is compromised in the presence of the p38 inhibitor. In *Xenopus* egg extracts, where MAPK, not p38, is responsible for the checkpoint, SB203580 did not inhibit the checkpoint function (*10*), suggesting that the drug appears not to affect components of the spindle assembly checkpoint other than p38.

Microinjection of XCL100, a phosphatase that dephosphorylates and inactivates MAPK and p38, into *Xenopus* tadpole cells abrogates the normal spindle assembly checkpoint (*8*). This result, together with our findings, indicates that activity of p38 is required for checkpoint function in somatic cell cycles.

Studies in yeast have revealed several components of the spindle assembly checkpoint, such as *MAD* and *BUB* genes (*7*). The recent identification of *Xenopus* and human homologs of *MAD2* indicates that the mechanism of the spindle assembly checkpoint is conserved evolutionarily from yeast to vertebrates (*16*). Because MAPK takes part in the cytostatic factor arrest of unfertilized *Xenopus* eggs (*3*, *4*) and active MAPK or p38 can induce mitotic arrest in cleaving embryos (*3*) and in in vitro cell cycles (*6*), a common target for MAPK and p38 might contribute to mitotic arrest.

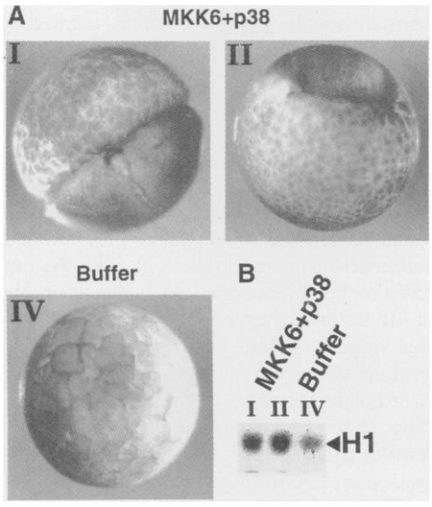

Fig. 4. Mitotic arrest of cleaving embryos induced by activated p38. (**A**) Activated p38 with MKK6 (I and II) or control buffer (IV) was microinjected into one blastomere of *Xenopus* embryos at the two-cell stage (*24*). The embryos were cultured at room temperature for 4 hours and photographed. (**B**) The embryos shown in (A) were lysed and assayed for histone H1 kinase activity.

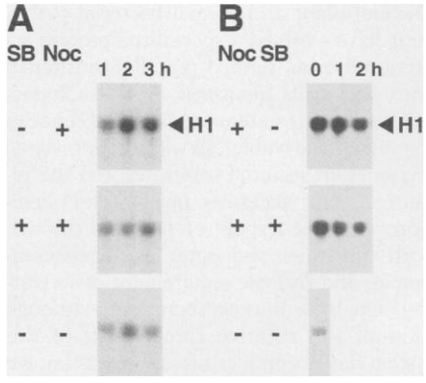

Fig. 5. Suppression of nocodazole-induced MPF activation by a specific inhibitor of p38. (**A**) NIH 3T3 cells were deprived of serum and released into the cell cycle by addition of serum. After 17 hours, SB203580 (SB, 20 μM) was added, and nocodazole (Noc) was added 3 hours later. At the indicated times after the addition of nocodazole, cell lysates were prepared and histone H1 kinase activities were measured. (**B**) NIH 3T3 cells were deprived of serum and released into the cell cycle by addition of serum. Nocodazole was added 20 hours after the addition of serum, and SB203580 (20 μM) was added 4 hours later. Cell lysates were prepared at the indicated times after the addition of SB203580 and histone H1 kinase activities were measured.

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14. In these experiments, we added or injected activated p38 together with MKK6 to maintain the p38 in an active state. Phosphorylated, activated p38 alone was not sufficient to induce the mitotic arrest. MKK6 did not cause arrest of the cell cycle in M phase (*10*).
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17. Recombinant MKK6 and recombinant p38 proteins were expressed in *E. coli* as described [T. Moriguchi *et al.*, *J. Biol. Chem.* **271**, 13675 (1996)]. The p38 protein (1 μg) with or without 0.5 μg of MKK6 protein was incubated for 15 min at 30°C in the presence of 100 μM adenosine triphosphate (ATP) and 15 mM $MgCl_2$. ATF2 (5 μg) and [γ-^{32}P]ATP (1 μCi) were then added to the mixture, which was incubated for 10 min. Phosphorylated ATF2 was detected by electrophoresis and autoradiography.
18. NIH 3T3 mouse fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) containing calf serum (CS, 10%). Cells were seeded onto 225-cm^2 flasks and allowed to grow for 3 days. Cells were cultured in DMEM containing CS (0.5%) for 30 hours to synchronize cells in G_0. Fresh DMEM supplemented with 10% CS was then added to induce the cells to reenter the cell cycle. After 18 hours, nocodazole (0.4 μg/ml) was added before cells entered M phase. Mitotic cells were collected 4 hours later by mechanical shake-off, washed with phosphate-buffered saline (PBS), and lysed in 50 mM Hepes (pH 7.4), 100 mM NaCl, 1 mM EGTA, 20 mM NaF, 20 mM sodium pyrophosphate, 1 mM sodium vanadate, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100. Cells remaining on flasks were scraped and lysed in the same buffer.
19. Extract (5 μl) was incubated with histone H1 (5 μg) in a final volume of 15 μl in the presence of 100 μM ATP, 5 μCi of [γ-^{32}P]ATP, and 15 mM $MgCl_2$ for 10 min at 30°C. The reactions were terminated by the addition of SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer. After boiling, the samples were subjected to SDS-PAGE and autoradiography.
20. Samples were incubated with anti-p38 (antibody to a COOH-terminal peptide, 20 μl; Santa Cruz) and 20 μl of protein A–Sepharose in the presence of 0.1% SDS for 2 hours at 4°C. The beads were washed three times with tris-buffered saline [20 mM tris-HCl (pH 7.5), 150 mM NaCl] containing 0.05% Tween 20. For immune-complex kinase assays, immunoprecipitated beads were incubated with recombinant ATF2 protein (5 μg) in a final volume of 20 μl in the presence of 100 μM ATP, 5 μCi [γ-^{32}P]ATP, and 15 mM $MgCl_2$ for 30 min at 30°C. After electrophoresis, radioactivity was detected by autoradiography or analyzed with an image analyzer (BAS2000, Fuji Photo Film). Similar results were obtained with an antibody to recombinant p38 that we produced.
21. MAPK was immunoprecipitated with anti-MAPK [Y. Gotoh *et al.*, *EMBO J.* **10**, 2661 (1991)], and the kinase activity was determined with myelin basic protein (MBP; 0.3 mg/ml) as a substrate in the presence of 100 μM ATP, 5 μCi of [γ-^{32}P]ATP, and 15 mM $MgCl_2$ for 30 min at 30°C. JNK was immunoprecipitated with an antibody to a COOH-terminal peptide of JNK (Santa Cruz) and assayed as described [T. Moriguchi, H. Kawasaki, S. Matsuda, Y. Gotoh, E. Nishida, *J. Biol. Chem.* **270**, 12969 (1995)].
22. *Xenopus* egg extracts were prepared as described [A. W. Murray, *Methods Cell Biol.* **36**, 573 (1991)]. Recombinant GST (gluthathione *S*-transferase)–p38 and histidine-tagged MKK6 proteins were added to the extracts at a final concentration of 0.1 mg/ml each.
23. Recombinant GST-p38 protein was incubated with or without recombinant MKK6 protein (*17*). Ste11ΔN was expressed in *E. coli* as described (*6*). Recombinant proteins were then added to the cell cycle extracts (final concentration, 0.1 mg/ml).
24. Samples (10 nl) were microinjected into one blastomere of the two-cell embryo as described (*4*).
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Supramolecular Structure of the *Salmonella typhimurium* Type III Protein Secretion System

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The type III secretion system of *Salmonella typhimurium* directs the translocation of proteins into host cells. Evolutionarily related to the flagellar assembly machinery, this system is also present in other pathogenic bacteria, but its organization is unknown. Electron microscopy revealed supramolecular structures spanning the inner and outer membranes of flagellated and nonflagellated strains; such structures were not detected in strains carrying null mutations in components of the type III apparatus. Isolated structures were found to contain at least three proteins of this secretion system. Thus, the type III apparatus of *S. typhimurium*, and presumably other bacteria, exists as a supramolecular structure in the bacterial envelope.

Several plant and animal bacterial pathogens have evolved a specialized protein secretion system, termed type III, to interact with host cells [reviewed in (*1*)]. Characteristics of this system are (i) the absence of a typical, cleavable, *sec*-dependent signal sequence in secreted substrates; (ii) the requirement of accessory proteins for secretion; (iii) the export of proteins through both the inner and outer bacterial membranes; and (iv) the requirement of activating signals to initiate secretion. Although most of the putative components of this system have been identified, little is known about their function or their organization in the bacterial envelope. Genetic analyses have established that these systems are both structurally and functionally conserved across bacterial species (*2*).

The human pathogen *Salmonella typhimurium* encodes two type III secretion systems, although only one of them, located at centisome 63 of its chromosome, appears to be expressed in vitro (*3*). This system directs the translocation of several bacterial proteins into the host cell (*4*), which activate host cell signaling pathways, leading to a variety of responses, such as reorganization of the actin cytoskeleton, cytokine production, and the induction of programmed cell death in macrophages (*1*). This system has also been associated with the assembly of invasomes, appendage-like structures that appear on the bacterial surface upon contact with host cells (*5*). Some of the putative components of the secretion apparatus share sequence homology with proteins of the flagellar export machinery, suggesting an evolutionary relation.

The similarity between type III secretion components and the flagellar export machinery prompted us to investigate whether the *S. typhimurium* cell envelope contains structures similar to those involved in flagellar assembly. A mutant strain with a deletion in the *flh*C gene and therefore lacking all flagellar proteins was osmotically

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