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Disruption of the Serine Proteinase Gene (*sep*) in *Aspergillus flavus* Leads to a Compensatory Increase in the Expression of a Metalloproteinase Gene (*mep20*)

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The serine proteinase gene (*sep*) in *Aspergillus flavus* was disrupted by homologous recombination with a hygromycin resistance gene as the marker. The gene-disrupted mutant GR-2 contained a single-copy insertion of the marker gene and did not express the *sep* gene. Serine proteinase activity, 36-kDa protein labeled by ^{3}H-diisopropylfluorophosphate, and immunologically detectable proteinase were not detected in the culture fluid of GR-2. Despite the absence of the serine proteinase, the total elastinolytic activity levels in the mutant and the wild-type *A. flavus* were comparable. Immunoblots revealed that the mutant secreted greater amounts of an elastinolytic metalloproteinase gene (*mep20*) product than did the wild type. Furthermore, *mep20* mRNA levels, measured by RNase protection assay, in the mutant were higher than those in the wild type. Inhibition of the serine proteinase by *Streptomyces* subtilisin inhibitor (SSI) in the culture medium of wild-type *A. flavus* also resulted in an elevation of *mep20* gene products. Although no serine proteinase activity could be detected, the level of elastinolytic activity of the SSI-treated culture was comparable to that of the control. Immunoblots revealed that the addition of SSI caused an elevation in the levels of metalloproteinase and its mRNA. These results suggest that the expression of the genes encoding serine and metalloproteinases are controlled by a common regulatory system and the fungus has a mechanism to sense the status of extracellular proteolytic activities.

Extracellular hydrolases are produced by many microbes to degrade and obtain nutrients from polymers found in their environment. In addition, microbial proteinases have been implicated in the colonization and virulence of a number of bacteria and fungi (9, 12, 21, 30, 32). In the invasive lung infections of immunocompromised hosts, aspergilli must penetrate proteinaceous barriers such as elastin, collagen, and laminin (45), and thus proteinases are thought to be virulence factors (5, 20, 27, 35, 37, 44). *Aspergillus fumigatus* and *A. flavus* produce serine proteinase and metalloproteinases that are capable of hydrolyzing such polymers (19, 25, 28, 33–37). A serine proteinase is produced as a major extracellular protein when these fungi are grown in a medium containing either elastin or collagen as the sole nitrogen source (19, 20, 35, 36). The serine proteinase genes (*sep*) in both fungi show about 80% amino acid sequence identity (15, 33). In addition, *A. fumigatus* produces a 42-kDa metalloproteinase and *A. flavus* produces a 23-kDa metalloproteinase (25, 28, 34, 36, 42). In *A. flavus*, the levels of the 23-kDa metalloproteinase and serine proteinase are almost comparable (33, 34, 36), while in *A. fumigatus*, the 42-kDa metalloproteinase is produced in amounts less than those of the serine proteinase (25, 28). The gene for the 23-kDa metalloproteinase in *A. flavus* would encode a protein with a theoretical molecular mass of 20 kDa, and, therefore, this gene was designated *mep20* and the protein was designated MEP20 (34). A homologous *mep20* found in *A. fumigatus* is poorly expressed (34). No protein homologous to the 42-kDa metalloproteinase of *A. fumigatus* was detected in the culture medium of *A. flavus* (34). Recently, gene-disrupted mutants of *A. fumigatus* have been developed by homologous recombination by using antibiotic resistance genes as markers (14, 29, 46, 47). Disruption of either the serine proteinase gene and/or the 42-kDa metalloproteinase gene in *A. fumigatus* failed to prevent mortality in immunocompromised mice (14, 29, 47). If the disruption of individual proteinase genes were accompanied by compensatory increases in the expression of other proteinase genes, the role of proteinases in invasive aspergillosis could not be defined. However, such compensatory changes in gene expression have not been reported. In this paper, we report that in *A. flavus*, the absence of serine proteinase activity in the medium due to gene disruption or the presence of a potent, specific serine proteinase inhibitor results in a compensatory increase in the level of the *mep20* gene transcripts and extracellular metalloproteinase activity.

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MATERIALS AND METHODS

Microorganism and growth medium. *A. flavus* 28 was grown in yeast-carbon base (YCB) medium with elastin as the sole nitrogen source (19, 33) in Roux bottles (100 ml of medium per bottle) at 37°C. For enzyme assays and analysis, 1 to 3 ml of the culture supernatant was drawn under aseptic conditions and stored at −80°C until analysis was done. For comparative studies, equal numbers (5×10^{6} for 100 ml medium) of same-age spores of the wild type and mutants were used.

Vector construction using hygromycin resistance gene marker and transformation of *A. flavus*. The plasmid pLA15 carrying the *sep* gene in a 2.95-kb *Xba*I genomic DNA fragment (33) was digested with *Sfi*I that cleaves in the open reading frame (ORF) of the gene at 1143 bp. A 2.3-kb DNA fragment carrying the *hph* gene with fungal promoter and terminating sequences for the expression of hygromycin resistance was isolated from the plasmid pBluescript KS CocHyg (1, 43) by digestion with *Bam*HI. The ends of the *Bam*HI fragment and the *Sfi*I-digested plasmid pLA15 were filled by Klenow polymerase, and the blunt ends were ligated. The resulting plasmid (pSPD1), carrying the *hph* marker gene in the ORF of the serine proteinase gene, was used to transform wild-type *A. flavus*. Approximately 5 μg of plasmid DNA was added to 10^{7} protoplasts as previously described (1, 43). After transformation, protoplasts were plated onto 1% glucose–0.5% yeast extract–1% Bacto Peptone (GYP)–1.2 M sorbitol agar plates containing 200 μg of hygromycin per ml. Fungal colonies growing on these plates were subcultured on minimal salts-glucose agar plates without hygromycin and then transferred back to GYP-hygromycin plates. Cultures from the GYP-

hygromycin plates were grown in 5 ml of YCB-elastin medium for 5 days, and culture supernatants were assayed for serine proteinase activity (see below). Mutants which lacked serine proteinase activity were repeatedly cultured on agar plates and checked for stability, as indicated by a lack of serine proteinase activity.

Analysis of mutants by Southern and Northern (RNA) blotting. Genomic DNA isolated from fungal cultures was digested with different restriction enzymes and analyzed by Southern hybridization using the ^{32}P-labeled *A. flavus sep* gene or *mep20* cDNA as probes, as reported previously (33, 38). For Northern blots, total RNA (15 μg) isolated from 108-h-old mycelia of the wild type and the mutant grown in the YCB-elastin medium was used. RNA was separated on the formaldehyde-agarose gels, and the standard protocols (38) for hybridization were used.

Immunoblotting and quantitation of protein. Protein was collected from culture supernatants at different periods of growth, precipitated with trichloroacetic acid (final concentration, 5%), washed with acetone, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels. For immunoblot analysis, protein was electroblotted (4, 33, 38) and antisera (1:500 dilutions) to serine proteinase and the 23-kDa metalloproteinase of *A. flavus* were used with ^{125}I-labeled protein A for detection. Whenever two proteinases were detected in the same sample, the blotted membrane was treated with antibodies to one protein, washed with phosphate-buffered saline, blocked for 1 h, and incubated with antibodies to the second protein. The protein levels were compared by quantitating the radioactivity in each band by using a PhosphorImager as described below for the RNase protection assays.

Labeling of serine proteinases with ^{3}H-DFP. Total protein (50 μg) was incubated with 70 μCi of ^{3}H-diisopropylfluorophosphate (^{3}H-DFP) for 60 min at room temperature, precipitated with trichloroacetic acid, and subjected to SDS-PAGE (22, 33). After electrophoresis, the gel was stained, destained, treated with Fluorohance (Research Products International Corp.; Mount Prospect, Ill.) according to the manufacturer's instructions, dried on chromatography paper, and exposed to the X-ray film at −80°C for several days.

Enzyme assays. The elastinolytic activity of the culture medium was measured with tritiated elastin in Tris-HCl buffer, pH 8.0, as reported previously (19, 33). Succinyl-Ala-Ala-Pro-Leu-paranitroanilide (*N*-Suc-Ala-Ala-Pro-Leu-*p*NA) was used as substrate to measure the serine proteinase activity (19). A fluorogenic substrate, Boc-Val-Leu-Lys-7,amido-4,methylcoumarin (Sigma Chemical Co., St. Louis, Mo.), was also used to assay the proteinase activity as reported (48), with some modifications. The reaction mixture containing 100 μl of enzyme solution, 0.33 mM substrate, and 60 mM sodium acetate buffer, pH 6.0, in 300 μl (total volume) was incubated at room temperature for 30 min. The reaction was terminated by adding 300 μl of 0.1 M trichloroacetic acid in 0.1 M sodium acetate buffer, pH 6.0. The final volume was adjusted to 2 ml with distilled water. The fluorescence of the released coumarin was measured in a fluorimeter with excitation at 370 nm and emission at 460 nm. The enzyme solution in the buffer was incubated with the inhibitor for 20 min before addition of the substrate for inhibitor studies.

Quantitation of *mep20* mRNA by RNase protection assays. Total RNA was isolated from the wild-type *A. flavus* and the mutant GR-2 that were grown under the same conditions in the YCB-elastin medium (19, 33). For RNase protection assays, total RNA preparations isolated from 60-, 84-, 108-, and 120-h-old mycelia were used. All RNA samples (about 10 μg in 10 μl) were treated with RNase-free DNase I (Bethesda Research Laboratories, Inc., Bethesda, Md.) at room temperature for 30 min, and the enzyme was heat inactivated at 65°C. About 1 μg of total RNA was used for each sample at each time point for obtaining comparative levels of the target molecules. A 282-bp PCR fragment generated from the *A. flavus mep20* gene (34) with the degenerate primers for the N terminus and the putative active-site amino acids (FQEEY, HEFTHA) of the protein, cloned into a pCR-II vector (Invitrogen, San Diego, Calif.), was used for making an antisense probe. The plasmid (1 μg) carrying the 282-bp *mep20* fragment was linearized with *Xba*I, treated with proteinase K for 30 min at 60°C, and purified by phenol-chloroform extraction twice. Antisense probe synthesis and the RNase protection assay were done with MAXISCRIPT and RPA-II kits, respectively (Ambion, Austin, Tex.). The antisense transcript (about 410 bp including some of the plasmid-derived oligonucleotide sequences) was prepared with SP6 phage RNA polymerase and 60 μCi of ^{32}P[CTP] (RNA label grade; 20 mCi/ml; Amersham, Arlington Heights, Ill.). After RNase digestion, the length of the protected fragment was 282 bp. The labeled probe was treated with DNase I to remove template DNA and separated on a denaturing polyacrylamide gel to remove the smaller fragments and the excess isotope; 8×10^5 cpm of the labeled probe was used for hybridization with each RNA sample. Yeast RNA was used as the control template. Hybridization was done at 45°C for 24 h with excess of the labeled DNA probe, and the unprotected fragments were degraded by RNase digestion according to the manufacturer's instructions. The hybridized RNA was separated on a 5% denaturing polyacrylamide gel. The dried gel was exposed to the Phosphor-screen (Kodak, Rochester, N.Y., and the radioactivity in the bands was quantitated by volume integration, with subtraction of the local background by a PhosphorImager (Molecular Dynamics Inc., Sunnyvale, Calif.) using the software ImageQuant. Values obtained as pixel density units were directly used for comparison.

Inhibition of serine proteinase in wild-type *A. flavus* culture medium by SSI. The *Streptomyces* gene encoding *Streptomyces* subtilisin inhibitor (SSI) was cloned and expressed in *Pichia pastoris*, and the protein was purified (24, 39). SSI was added to 2 μM after 27 and 72 h of growth in YCB-elastin medium. Aliquots (1.5 ml) of the medium were withdrawn at 24-h intervals, up to 120 h of growth. The culture supernatant was used for enzyme assays and immunoblotting, as described above. Total RNA (1 μg) isolated from 120-h-old mycelia grown with and without addition of the inhibitor was used for RNase protection assays as described above.

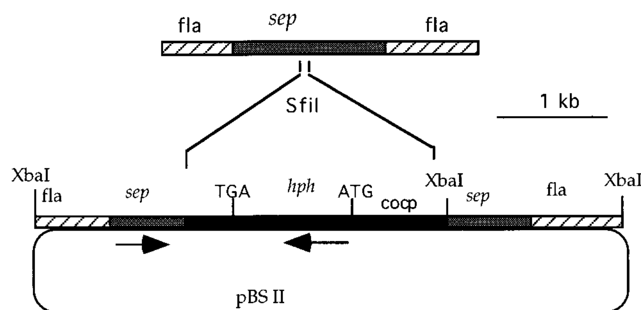

FIG. 1. Transformation vector used for serine gene disruption. *hph*, *Escherichia coli* hygromycin B resistance gene; cocp, *Cochliobolus heterostrophus* promoter; *sep*, serine proteinase ORF; fla, flanking region.

RESULTS

Transformation of *A. flavus*. The plasmid pLA15 carries a 2.95-kb *Xba*I genomic fragment of *A. flavus* containing the *sep* gene with approximately 700 bp of upstream and 850 bp of downstream flanking DNA (Fig. 1). The *sep* gene ORF was disrupted by inserting a 2.3-kb hygromycin resistance gene, yielding plasmid pSPD1 with 1.3 and 1.6 kb of *A. flavus* genomic DNA on either side of the marker gene. An additional *Xba*I site in pSPD1 was created at one of the ligating ends during the insertion of the hygromycin resistance gene. A high level of resistance of wild-type *A. flavus* to hygromycin was reported as a major problem in using this antibiotic for selection (51). We experienced the same problem with minimal salts-hygromycin medium; however, when GYP-sorbitol medium containing hygromycin instead of minimal salts medium was used for regenerating protoplasts, the background was negligible. Stable transformants expressing hygromycin resistance were selected by repeated subculturing from single colonies. All the mutants were grown in the minimal salts-glucose medium to eliminate potential auxotrophic mutants. The transformation frequency with the plasmid pSPD1 was 10 to 50 transformants per μg of DNA.

Analysis of mutants. Mutants that were unable to hydrolyze *N*-Suc-Ala-Ala-Pro-Leu-*p*NA were selected for further analysis. Mutants GR-2 and GR-8 showed no activity after repeated subculturing, while others with initial low levels of serine proteinase activity slowly recovered the activity level in the medium. Immunoblots of the culture filtrates with anti-serine proteinase antibodies showed that the two mutants did not produce this enzyme. Southern blots were probed with the labeled *sep* gene to analyze the integration of the marker DNA into the mutant genomes (Fig. 2). *Xba*I-digested genomic DNA of wild-type *A. flavus* showed hybridization to a 3-kb fragment, while the GR-2 mutant showed a major band of 3.8 kb and a minor band at 1.6 kb plus two more bands whose size would depend on the integration site of the vector and the genomic DNA. GR-8 showed multiple hybridizing bands. Southern blots of the genomic DNA digested with *Sph*I, which has no sites in pSPD1, showed hybridizing bands of about 12.7 kb in GR-2, as expected from the 8.3-kb vector plus the size of the hybridizing *Sph*I genomic fragment. Again multiple bands

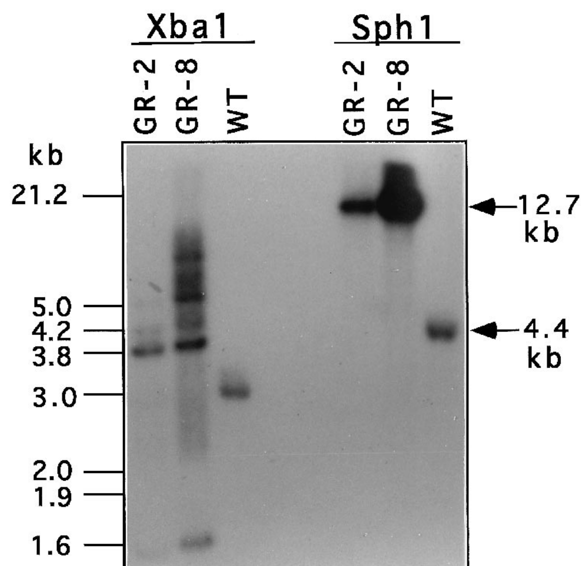

FIG. 2. Southern blot of *Xba*I- and *Sph*I-digested genomic DNA isolated from *A. flavus* wild type (WT) and *sep* gene-disrupted mutants GR-2 and GR-8. ^{32}P-labeled *sep* gene was used as the probe.

were observed with GR-8, while the wild-type showed a 4.4-kb hybridizing fragment. The larger size of the hybridizing bands in the mutants indicates that plasmid pSPD1 with the hygromycin resistance marker and *sep* gene flanking regions integrated at the homologous region in the genome. In GR-2, a single copy of the vector integrated, while in GR-8, multiple copies of the vector integrated (Fig. 2); therefore, GR-2 was selected for further studies. RNA blots of the total RNA isolated from the GR-2 and the wild-type *A. flavus*, probed with the ^{32}P-labeled serine proteinase gene, showed the absence of the *sep* gene transcripts in this mutant (data not shown). SDS-PAGE of the extracellular proteins showed that the serine proteinase band that was readily found in the wild type as a major Coomassie-staining 36-kDa band was missing in the case of GR-2 (Fig. 3). Growth rate, spore formation, colony morphology and other characteristics of the mutants were indistinguishable from those of the wild-type *A. flavus* strain.

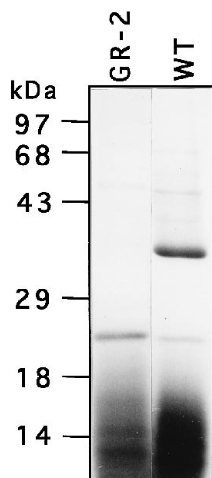

FIG. 3. SDS-PAGE of extracellular proteins of *A. flavus* wild type (WT) and GR-2 grown in YCB-elastin medium. Protein precipitated with trichloroacetic acid from equal volumes of culture filtrate from 5.5-day-old cultures was separated on 12% gel.

Measurement of serine proteinase and elastinolytic activities in culture medium of mutant and wild-type strains. In the culture filtrates of the wild-type fungus, measurable hydrolysis of *N*-Suc-Ala-Ala-Pro-Leu-*p*NA was readily detectable (data not shown), whereas in the culture filtrate of GR-2, hydrolysis of this substrate could not be detected even after 2 h of incubation. After overnight incubation of the reaction mixture, a very low level of hydrolysis was detected. The fluorogenic substrate Boc-Val-Leu-Lys-7,amido-4,methylcoumarin was reported to be hydrolyzed by the 23-kDa metalloproteinase of *A. oryzae*, which is homologous to the *A. flavus* protein MEP20 (34, 48). We initially selected this substrate to monitor the levels of the MEP20 in *A. flavus*. However, culture filtrates of GR-2, which contained no serine proteinase detectable by immunoblotting but contained elevated levels of the metalloproteinase, did not hydrolyze this substrate. With this assay, serine proteinase activity was barely detectable in the culture fluid of GR-2 whereas in the wild type the enzyme activity increased up to 5 days of growth (Fig. 4A). That the activity cleaving this fluorogenic substrate in the culture fluid of the wild type was due to serine proteinase was confirmed by the observation that 1 mM phenylmethylsulfonyl fluoride (PMSF) completely inhibited the substrate hydrolysis whereas 1 mM phenanthroline (metalloproteinase inhibitor) had no effect (Fig. 4A). Thus, the fluorogenic substrate we used is specific for the serine proteinase.

We tested whether the lack of serine proteinase in GR-2 was manifested in a decreased elastinolytic capability of the extracellular fluid. The culture fluid of the mutant GR-2 and the wild-type fungus showed the same level of elastinolytic activity with ^{3}H-elastin as the substrate (Fig. 4B). In both cases, the highest level of activity was detected after 72 h of growth, with some decrease in activity during subsequent days. That a serine proteinase was not involved in the elastinolytic activity was shown by the observation that PMSF did not inhibit the total elastinolytic activity of the mutant (data not shown).

Test for other serine proteinases in culture fluids of GR-2 and wild-type *A. flavus*. To identify the number of serine proteinases produced by the wild type and GR-2, we incubated the total extracellular protein from 5.5-day-old cultures with ^{3}H-DFP, the serine proteinase-specific inhibitor that reacts with the active-site serine residues (26). Autoradiography after SDS-PAGE (Fig. 5) revealed only one dark band in the culture fluid from the wild-type strain, which corresponded to the serine proteinase band on the Coomassie blue-stained gel. In the mutant, there was no band at the corresponding position, but a longer exposure showed a very faint band corresponding to a size slightly larger than the major serine proteinase. This protein appears to be produced in very small amounts by the mutant GR-2. This faint band is also probably present in the wild-type fungus but is masked by the large amount of serine proteinase that is labeled by the ^{3}H-DFP; the absence of this major protein revealed the other ^{3}H-DFP-labeled protein in the mutant. On the stained SDS-PAGE gels, the dominant serine proteinase band seen in the culture medium of the wild-type fungus was absent in GR-2. Furthermore, immunoblot analysis using anti-serine proteinase antibody showed that levels of the 36-kDa protein increased in culture fluids of the wild-type strain up to 144 h whereas this protein was absent in extracellular fluids of GR-2 (Fig. 6A).

Effect of serine proteinase gene disruption on metalloproteinase levels in culture medium. Since *sep* gene disruption did not cause a measurable decrease in elastinolytic activity, we tested whether there was a compensatory increase in the elasti-

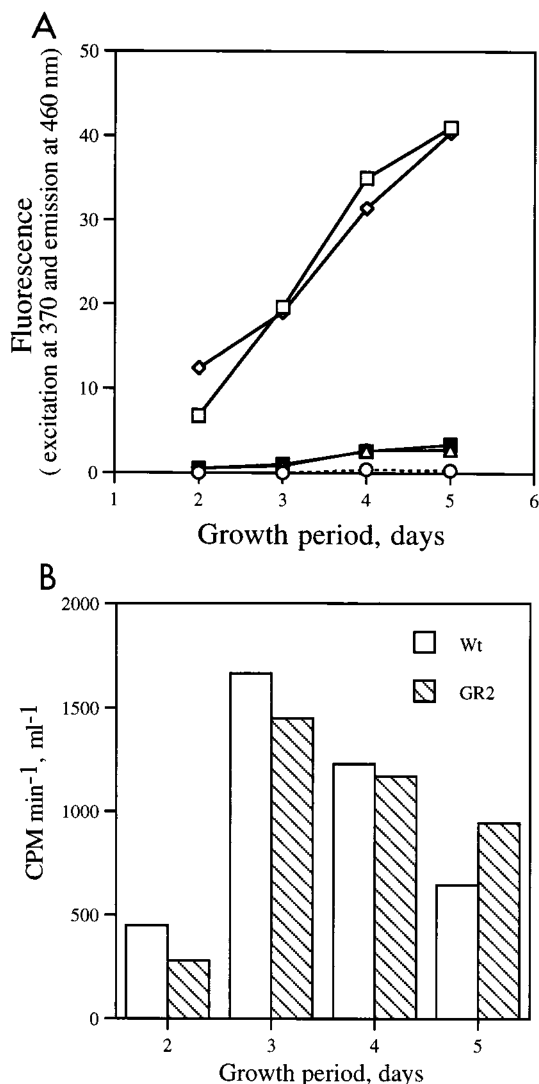

FIG. 4. (A) Effects of treatment of culture filtrates of *A. flavus* wild type and GR-2 with PMSF and 1,10-phenanthroline on hydrolysis of the fluorogenic substrate Boc-Val-Leu-Lys-7,amido-4,methylcoumarin. □, phenanthroline-treated wild type; ○, PMSF-treated wild type; ◇, untreated wild type; △, phenanthroline-treated GR-2; ■, untreated GR-2. PMSF treatment of GR-2 had no effect. (B) Total elastinolytic activity in culture filtrates of *A. flavus* wild type (Wt) and GR-2, measured with ^{3}H[elastin] as substrate.

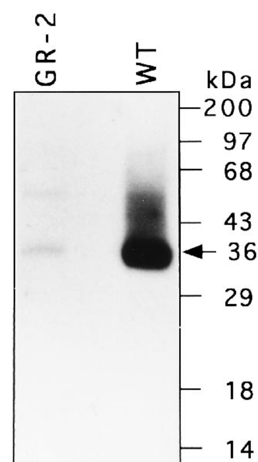

FIG. 5. Autoradiogram of SDS-PAGE of ^{3}H-DPF-treated total extracellular protein of *A. flavus* wild type (WT) and GR-2.

nolytic metalloproteinase level in the culture fluid of the mutant by immunoblot analysis with MEP20 antibody. The level of this protein increased to comparable levels in both wild-type and mutant culture fluids up to 96 h of growth (Fig. 6A and B). After 96 h, the amount of the protein in the wild type decreased whereas with GR-2 the metalloproteinase level continued to increase even up to 144 h. In contrast, the serine proteinase level remained the same later than 120 h in the wild-type *A. flavus*. With GR-2, a faint cross-reacting band close to the 36-kDa protein appeared very late in the growth phase, after 96 h. This cross-reacting protein matches with that labeled by ^{3}H-DFP (Fig. 5). Ectopic integration of pSPD1 in two mutants (without *sep* gene disruption) did not cause compensatory increases in metalloproteinase levels (data not shown). With the other *sep* gene-disrupted mutant (GR-8), total elastinolytic activity of the extracellular fluid was unchanged, and SDS-PAGE of ^{3}H-DFP-treated extracellular fluid did not reveal ^{3}H-labeled 36-kDa protein and showed increased levels of metalloproteinase (data not shown). Thus, in this mutant also *sep* gene disruption was accompanied by a compensatory increase in MEP20 production.

Measurement of *mep20* mRNA levels in wild type and GR-2. To test whether increased metalloproteinase production in the *sep* gene-disrupted mutant was due to increased *mep20* transcript levels, this mRNA was measured by RNase protection assays. A 282-bp *mep20* gene fragment from within the ORF was cloned by PCR (34) and used as the template for making an antisense probe. The advantage of using part of the ORF is that even partly degraded mRNA with an intact region complementary to the probe can be detected. The total length of the probe was about 410 bp, and the size of the RNase-protected fragment would be about 282 bp. Such a difference in size would allow us to distinguish the RNase-protected fragments from the unprotected ones. The RNase protection assay showed the hybridized fragments of the expected size (Fig. 7A). With yeast RNA as the control template, no protection of the antisense probe was observed after RNase digestion, indicating the specificity of the probe. The metalloproteinase mRNA in the mutant was already at higher levels compared with that in the wild type during the early growth periods (Fig. 7B), and subsequently (72 to 96 h) the mRNA levels in the mutant reached much higher levels than that found in the wild type; later (120 h) the level of mRNA in the mutant decreased (Fig. 7A and B).

Effect of SSI in growth medium on metalloproteinase production by wild-type *A. flavus*. The increase in metalloproteinase levels in the *sep* gene-disrupted mutant indicated that the two proteinases might be under a common regulatory control. To test whether inhibition of serine proteinase activity in the culture medium of wild-type *A. flavus* would also lead to increased levels of metalloproteinase, as seen in the *sep* gene-disrupted mutant, we added SSI (39), which is known to inhibit the fungal serine proteinase (23, 24). In the culture that received the inhibitor, no serine proteinase activity was detected (Fig. 8A) although the protein was present in the medium as detected by immunoblots (data not shown). In the control that did not receive the inhibitor, we could detect serine proteinase activity (Fig. 8A). However, the total elastinolytic activity in both cases was almost the same and followed the same pattern of appearance in the medium during growth (Fig. 8B). Immunoblot analyses of the culture filtrate from the control and the

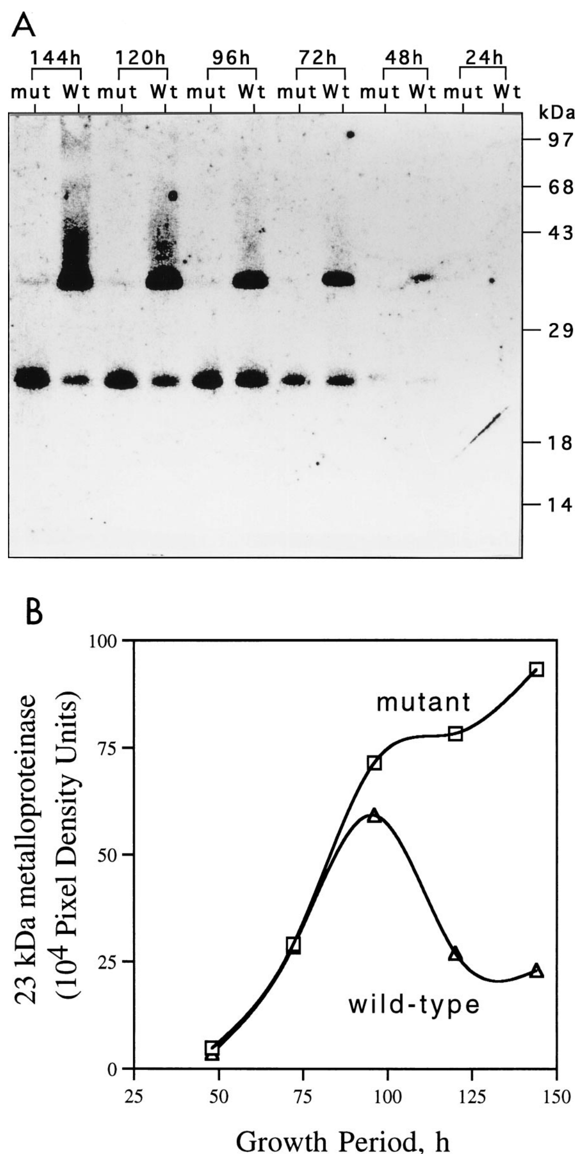

FIG. 6. Detection by immunoblotting of SEP and MEP20 secreted by *A. flavus* wild type (Wt) and *sep* gene-disrupted mutant GR-2 (mut), grown in YCB-elastin medium with the respective antibodies used sequentially. Protein precipitated from 200 μl of culture supernatant by trichloroacetic acid was subjected to SDS-PAGE and immunoblotting. The levels of radioactivity in bands labeled with ^{125}I-labeled protein A (A) were quantitated with a PhosphorImager (B).

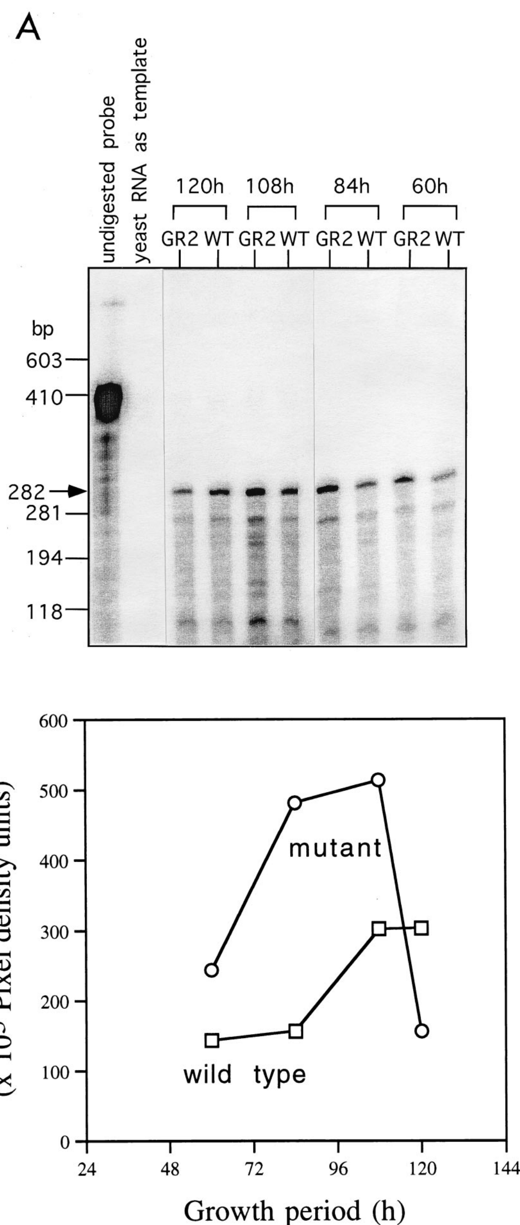

FIG. 7. Detection of *mep20* mRNA levels in wild-type (WT) *A. flavus* and *sep*-disrupted mutant GR-2 by RNase protection assay. The DNase I-treated total RNAs isolated at different growth periods from the cultures grown under identical conditions were used with a 410-bp ^{32}P-labeled antisense probe. The RNase-protected fragment should have 282 bp (A). Yeast RNA used as the experimental control template showed no hybridization. The *Hae*III-digested phage X-174 DNA was end labeled and used as size markers. (B) The radioactivity in each protected mRNA fragment was quantitated with a PhosphorImager, and the values were directly plotted for comparison.

serine proteinase-inhibited culture indicated that the levels of the metalloproteinase, although almost the same in both the cultures until 96 h and decreased in the control, increased in the culture in which the serine proteinase was inhibited (Fig. 8C). In addition, *mep20* mRNA levels were measured by an RNase protection assay using total RNA isolated from 120-h-old mycelia treated with and without SSI. The results showed that in 120-h-old cultures, the *mep20* mRNA level was threefold higher in the presence of inhibitor than in the untreated control (Fig. 9).

DISCUSSION

We constructed a serine proteinase gene-disrupted mutant of *A. flavus*, using hygromycin resistance as the selection marker. The mutant GR-2, with plasmid pSPD1 integrated at the homologous region in the genome, showed no expression of the *sep* gene. A typical substrate for human macrophage elastase, *N*-Suc-Ala-Ala-Val-Leu-*p*NA, is also hydrolyzed by the serine proteinase of aspergilli (19). We could not detect any serine proteinase activity in the culture filtrate of GR-2 using this substrate. The fluorogenic substrate Boc-Val-Leu-Lys-7,amido-4,methylcoumarin was also not hydrolyzed. To further test whether other serine proteinases are secreted by the gene-disrupted mutant, we treated the culture medium

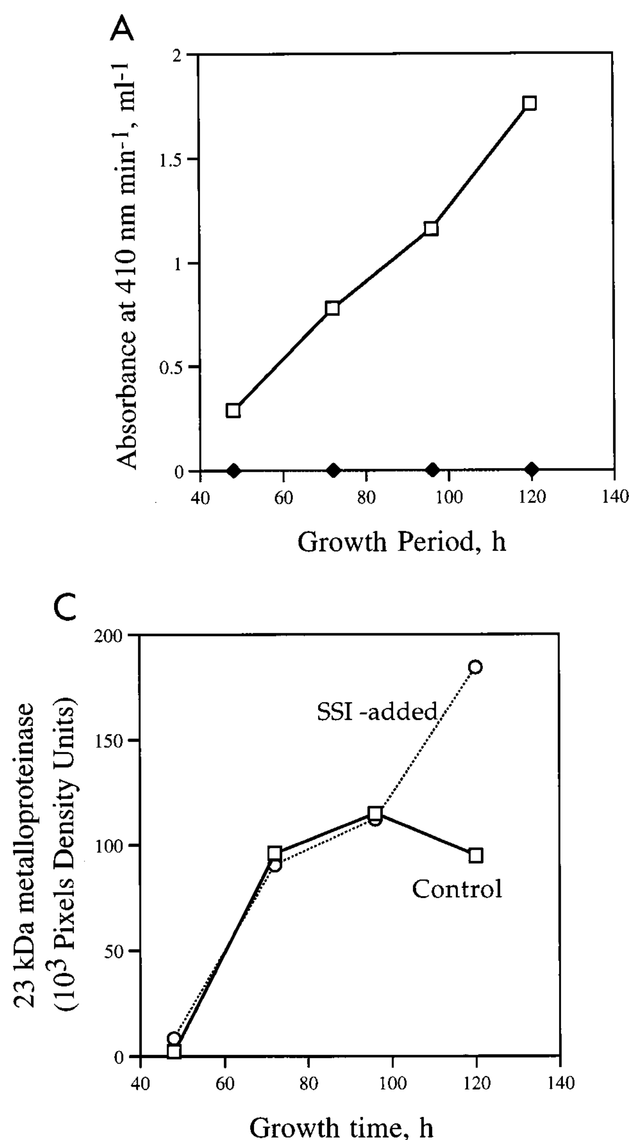

FIG. 8. (A) Serine proteinase activity in culture filtrate of wild-type *A. flavus* without (□) and in the presence of (◆) SSI. Proteinase activity was measured with *N*-Suc-Ala-Ala-Pro-Leu-*p*NA as the substrate. The fungus was grown in YCB-elastin medium. (B) Total elastinolytic activity of culture fluid of wild-type *A. flavus* grown without (control) and in the presence of SSI. The enzyme activity was measured with ^{3}H[elastin]. Bars, standard errors. (C) MEP20 levels in culture fluid of wild-type *A. flavus* grown with (○) and without (□) SSI in growth medium. Immunoblots with ^{125}I-labeled protein A were quantitated with a PhosphorImager.

with ^{3}H-DFP that reacts and covalently attaches to the active serine; only one protein corresponding to the 36-kDa serine proteinase was labeled in the wild-type *A. flavus*. This band was missing in GR-2, although a faint band, with a slightly greater molecular mass than the 36-kDa serine proteinase, could be detected on longer exposures of the X-ray film. After prolonged growth of the mutant (96 h) this band could also be detected in culture fluids with anti-serine proteinase antibody. This protein also corresponded with that weakly labeled with ^{3}H-DFP. This protein is probably responsible for the very low level of serine proteinase activity observed in the mutant after long incubation periods with the synthetic substrates. This protein is obviously encoded by a gene other than the one disrupted in the present experiment. Whether this gene is expressed when the fungus invades the hosts is not known.

Even though serine proteinase is a major proteinase produced by *A. flavus* (33), and despite the absence of this enzyme in the culture supernatant, GR-2 showed about the same level of elastinolytic activity as the wild type. A *sep* gene-disrupted *A. fumigatus* strain was reported to have a remarkably lowered level of elastinolytic activity and collagenolytic activity when elastin-Congo red, and azocollagen were used as substrates (46). On the other hand, *sep* gene-disrupted mutants of *A. fumigatus* developed by Monod et al. (29) showed significant residual activity on azocollagen (30% of the wild type) but not on elastin-Congo red. Their results also showed that in the PMSF-treated culture filtrate of the wild-type fungus, no elastinolytic activity was detected, but about 30% of the total azocollagen hydrolyzing activity was observed (29). The residual collagenolytic activity was attributed to the 42-kDa metalloproteinase that was reported not to be active on elastin (28). However, Markaryan et al. (25) showed that the 42-kDa metalloproteinase of *A. fumigatus* can also degrade elastin, but not as efficiently as the serine proteinase. A collagenolytic aspartic proteinase was also found in *A. fumigatus* (23). It appears that in *A. fumigatus*, the serine proteinase is the major extracellular proteinase that contributes to elastinolytic activity. In contrast, disruption of the serine proteinase-encoding gene in *A. flavus* did not result in any significant loss of elastinolytic activity. This observation prompted us to test for any compensatory increases in other elastinolytic proteinases, such as the 23-kDa metalloproteinase (36) produced by this fungus. Immunoblot analyses showed that metalloproteinase levels were elevated in the mutant GR-2. That this increase in MEP20 level results from *sep* gene disruption rather than some other mutagenesis arising from transformation was strongly suggested by the observation that two mutants with ectopic integration of the vector did not show compensatory increases in MEP20 activity, whereas both *sep* gene-disrupted mutants, GR-2 and GR-8, showed compensatory increases in MEP20. This increase in MEP20 level could be because of two reasons. If serine proteinase degrades the metalloproteinase, the absence of serine proteinase could increase the half-life of the extracellular metalloproteinase. However, protein degradation was not detected

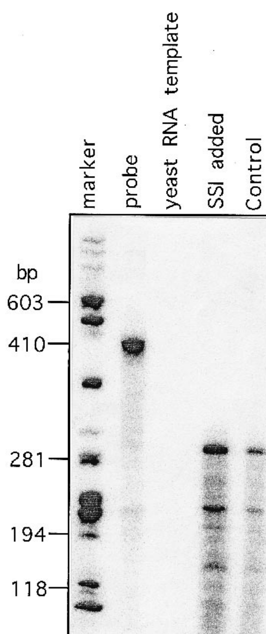

FIG. 9. Effect of inhibition of extracellular serine proteinase activity by SSI on *mep20* mRNA levels of wild-type *A. flavus*. SSI was added after 24 and 72 h of growth, and RNA was measured after 120 h of growth in YCB-elastin medium. The *mep20* mRNA was measured by RNase protection assaying, and the levels of radioactivity in the protected fragments measured with a PhosphorImager were 9.1×10^4 and 3.1×10^4 pixels for the sample with SSI added and the control, respectively.

in the immunoblots. The native mature metalloproteinase of *A. fumigatus* was found to be stable to proteolytic enzymes such as trypsin (24a). Alternatively, the absence of transcription of the *sep* gene may cause a compensatory increase in the expression of *mep20*. RNase protection assays clearly showed that the *mep20* mRNA levels in GR-2 were higher than in the wild type during the initial phase of growth and continued to increase up to 108 h. This may result from either a true increase in the transcription rate of the *mep20* gene or mRNA stabilization. Irrespective of the mechanism, serine proteinase gene disruption led to higher *mep20* mRNA and MEP20 protein levels. One possibility is that in the absence of *sep* gene transcription, higher levels of common factors were available for *mep20* gene transcription.

Elevated levels of metalloproteinase were also observed in the culture medium of SSI-treated wild-type *A. flavus*. Under such conditions, serine proteinase activity was completely inhibited, although normal amounts of immunologically cross-reacting serine proteinase were found. Treatment with SSI did not cause any significant difference in the total elastinolytic activity. The SSI-treated cultures of wild-type *A. flavus* showed increased levels of *mep20* mRNA and MEP20 protein in the culture medium, as was observed for the *sep* gene-disrupted mutant. For example, in the 120-h-old serine proteinase-inhibited culture, the MEP20 protein level was high and the *mep20* mRNA level was at least three times higher than in the control without inhibitor. The presence of SSI in the medium probably did not affect the expression of the serine proteinase gene, although proteinase activity could not be detected. Thus, absence of serine proteinase activity in the culture medium by gene disruption or enzyme inhibition caused the production of higher levels of *mep20* mRNA and MEP20 protein. These results suggest that the fungus can assess the extracellular proteinase status, perhaps by end product concentration, and accordingly regulate the expression of proteinase genes. It is also known that nutrient limitation in the growth medium triggers the expression of the genes encoding proteolytic enzymes in aspergilli (17). It is not known whether the same regulatory system can regulate the expression levels of different genes encoding functionally analogous proteinases. The response of *A. flavus*, of maintaining total proteinase activity by increasing the MEP20 levels when serine proteinase activity is either inhibited or abolished by gene disruption, suggests that the genes encoding the two proteinases are under a common regulatory control. It is also possible that genes encoding other proteinases may fall under the same regulatory system. For example, *Pseudomonas aeruginosa lasA* and *lasB*, genes encoding elastinolytic serine and metalloproteinases, respectively (31, 49), were reported to be regulated by a common regulator, homoserine lactone, and transcriptionally regulated by a repressor protein, LasR, and an inducer, LasI (50, 52). After inhibition of extracellular proteinase activity compensatory increases in the production of other proteinases is known to occur in animals. Insect larvae compensated for loss of tryptic activity caused by proteinase inhibitors in the diet by a 2.5-fold induction of a new proteinase activity that was insensitive to inhibition (16). Also, in rats and humans inhibitor-resistant proteinases were induced in response to the presence of the proteinase inhibitors (10, 11). A monitor peptide was reported to be involved in this compensatory induction (13). Whether analogous control mechanisms are involved in the fungal system is not known.

Virulence of *A. fumigatus* mutants disrupted in either the serine proteinase or metalloproteinase genes was reported to be similar to that of the wild type, and it was concluded that these proteinases were not virulence determinants for invasive pulmonary infection in immunocompromised mice (29, 47). However, with the double gene-disrupted *A. fumigatus* only 20% of the immunocompromised mice died in 24 h, whereas 70% of the mice inoculated with the wild type died during the same period, although most animals from both groups died later, suggesting a decrease in virulence. Although proteolytic activity was not detected at a neutral pH in the culture medium of the *A. fumigatus* double mutant, the expression of other proteinase genes, such as acid proteinase (23) and the *mep20* gene, (34) was not tested. We found that this double gene-disrupted mutant (provided to us by M. Monod) produced as much extracellular collagenolytic activity as did the wild type in assays with soluble collagen as substrate and the Folin reagent for free-tyrosine measurement (23, 37a). However, we do not know whether compensatory increases in other proteolytic enzymes occurred in *A. fumigatus* in response to gene disruption as seen with *A. flavus*. Multiple virulence determinants involved in host-parasite interaction have been reported for many bacteria (7, 26, 30, 52). The genes encoding virulence determinants appear to be coordinately regulated by common regulatory systems in response to the environmental signals (26), e.g., iron for diphtheria toxin production (3), osmolarity and pH for regulation of *toxR* and related genes in *Vibrio cholerae* (6), induction of the *Salmonella typhimurium* gene *invA* by anaerobic conditions (40), and regulation of virulence genes of *Yersinia pestis* by calcium (2). Our results also indicate that when there are multiple genes (12, 41, 50, 52) encoding different functionally analogous proteins, gene disruption experiments for studying the function of a particular gene may not give reliable information about their roles because other proteins may functionally substitute for the disrupted gene product. Furthermore, other virulence-associated genes may be expressed only in the host. Such is the case for some of the

extracellular hydrolytic enzymes that microbes use to degrade structural barriers in plants. For example, a phytopathogenic bacterium, *Erwinia chrysanthemi* (18), and the fungus *Fusarium solani pisi* (8) express several pectate lyase genes in culture but express unique pectate lyase genes exclusively in planta. Whether fungal extracellular proteinases are uniquely expressed in animal hosts and, if so, whether such proteinases are involved in invasive aspergillosis remain to be elucidated.

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