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Zinc-Regulated Biosynthesis of Immunodominant Antigens from *Aspergillus* spp.

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ASPND1 and ASPF2 are immunodominant antigens from *Aspergillus nidulans* and *A. fumigatus*, respectively, that are readily synthesized in infections in the human host, as demonstrated by their reactivity with more than 80% of sera from patients with aspergilloma or allergic bronchopulmonary aspergillosis. We demonstrate here that both antigens are exclusively produced under situations of low bioavailability of free Zn^{2+}. Addition of micromolar concentrations of Zn^{2+} to the culture medium strongly stimulated *Aspergillus* growth but totally inhibited ASPND1 or ASPF2 production. This effect was specific, since other divalent metals had no effect. Removal of endogenous Zn^{2+} by a chelator also stimulated ASPND1 production, and the effect was specifically reversed by Zn^{2+}. These results suggest a possible role of these antigens in the survival of the fungus in the lungs.

Several *Aspergillus* species are opportunistic pathogens causing respiratory diseases (aspergilloma and allergic bronchopulmonary aspergillosis [ABPA]) in normal hosts and invasive or disseminated infections in immunosuppressed patients (16). Immunocompetent affected individuals often have high levels of antibodies directed at fungal components. Although these antibodies may not always provide an effective defense against the fungus, they can be useful for diagnostic purposes.

ASPND1 is an *Aspergillus nidulans* immunodominant antigen that is highly reactive to sera from aspergilloma-affected individuals. Recent molecular cloning and characterization of the *aspnd1* gene (5) has revealed a high degree of homology to a well-characterized *A. fumigatus* allergen (ASPF2) which is consistently reactive to sera from individuals suffering from ABPA (1). Both cross-reactive antigens (of unknown function) have been overproduced in bacteria as recombinant proteins which retain the ability to react with both immunoglobulin G- and immunoglobulin E-specific antibodies. Characteristics common to the antigens are that they are detected only when the fungi are grown in certain conditions (especially in Czapek-Dox medium) and that they elicit a strong immune response (4, 15). These observations may reflect how the expression of these proteins is regulated in vivo and could provide some clues about their function, if any, as determinants of virulence.

In bacterial systems, virulence genes seem to be integrated into complex regulatory networks which determine the expression of virulence factors only when needed (19). In most cases, these regulatory circuits switch on in response to a few signals, such as a temperature of 37°C, iron deprivation, or contact with eukaryotic cells, which are all environmental conditions expected to be found in the bodies of mammals (6). In the case of *Aspergillus*, only iron deprivation has been shown to stimulate the synthesis of products involved in the survival of the invading fungus (3).

We report here that the factor responsible for the production of ASPND1 and ASPF2 is the lack of Zn^{2+} in the culture medium. We started our study with *A. nidulans*, observing that identical regulatory mechanisms are present in *A. fumigatus*

and other *Aspergillus* species. Our results are consistent with the idea that zinc deprivation could induce the in vivo synthesis of specific zinc-regulated fungal proteins in the human body. Zinc starvation could therefore be considered a new signal for fungal pathogens from the host environment.

MATERIALS AND METHODS

Organisms and growth conditions. *A. nidulans* G1059wt (*adF17 pabaA1 yA2*) was obtained from A. J. Clutterbuck, Glasgow, Scotland. Two different *A. fumigatus* isolates were used: ATCC 9197 (here after called *A. fumigatus* C), from the American Type Culture Collection and *A. fumigatus* R, a clinical isolate from the sputum of a patient with pulmonary aspergilloma. *A. flavus*, isolated from a lymphadenopathy in a patient with breast cancer and cutaneous invasive aspergillosis, was a gift from A. del Palacio (12 de Octubre Hospital, Madrid, Spain). *A. terreus* is a strain from the Spanish Type Culture Collection (CECT 2748).

The organisms were maintained on solid YED medium (1% [wt/vol] D-glucose, 1% [wt/vol] Difco yeast extract, 2% [wt/vol] agar). To obtain high yields of conidia, the fungi were grown on solid *Aspergillus* complete minimal medium (Amm) or AMM containing 1% glucose, 0.6% NaNO₃, 0.052% MgSO₄, 0.052% KCl, 0.15% KH₂PO₄, traces of FeSO₄ and ZnSO₄, and 1.5% (wt/vol) agar (pH 6.5). Plates were incubated at 28°C for at least 4 days.

For liquid growth cultures, four different media were used: YED, AMM, Bacto Sabouraud dextrose broth (SAB), and Bacto Czapek-Dox broth alone (CD) or mixed 1:1 with Bacto synthetic broth AOAC (CDA). SAB, CD, and CDA media were obtained from Difco Laboratories (Detroit, Mich.). For *A. nidulans*, AMM, SAB, CD, and CDA media, both solid and liquid, were supplemented with 10 mg of p-aminobenzoic acid and 200 mg of adenine per liter.

All *Aspergillus* species were grown by inoculation of 10⁵ conidia per ml in 1-liter Erlenmeyer flasks containing 300 ml of the corresponding liquid medium followed by incubation at 28 or 37°C in an Adolph Kühner orbital shaker at 250 rpm. Mycelia were harvested from liquid medium cultures by filtering through Whatman GF/C paper and washed thoroughly with double-distilled H₂O. The wet cake was immediately frozen and kept at −70°C until used.

Preparation of cell extracts. Frozen mycelia were thawed and mixed with lysing buffer (100 mM Tris-HCl [pH 7.5] containing 1 mM EDTA, 5 mM dithiothreitol, 1 mM freshly added phenylmethylsulfonyl fluoride [Sigma Chemical Co.], 5 µg of aprotinin per ml, and 5 µg of pepstatin A per ml [both obtained from Boehringer Mannheim]) to give a dense suspension. Samples were then disrupted in the 20,000-lb/in² cell of an SLM Aminco French press, previously refrigerated at −20°C, at a pressure of 16,000 lb/in². Complete breakage was monitored by microscopic observation. Sodium dodecyl sulfate (2%, final concentration) was added to the lysed mycelia, and the lysate was incubated for 10 min at 100°C. Clarified extracts (12,000 × g, 15 min) were aliquoted and stored at −70°C.

Protein was quantitated by a modification of the Lowry method (24). Extracts containing less than 1 mg of protein per ml were concentrated by precipitation with 7 volumes of cold acetone at −70°C for at least 3 h. Precipitated protein was pelleted by spinning for 20 min at 12,000 × g at 4°C, dried in a vacuum evaporator (Savant Instruments), carefully resuspended in 2% SDS to the desired concentration, and clarified by centrifugation at 3,000 × g 15 min.

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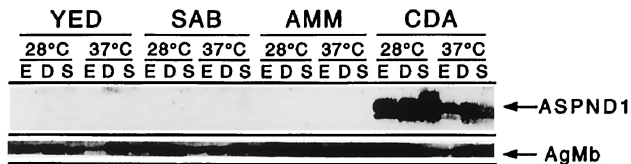
FIG. 1. Immunoblot of total mycelial extracts from *A. nidulans* cultured in different media (20 μg of protein/lane), separated on an SDS–14% polyacrylamide gel, and developed by using as the first antibody a monospecific anti-ASPND1 (ASPND1) or antimembrane (AgMb) rabbit serum at 1/500 dilution. Lanes: E, exponential phase; D, deceleration phase; S, stationary phase.

SDS-polyacrylamide gel electrophoresis (PAGE). Electrophoreses were carried out on a Protean II or Mini-Protean apparatus (Bio-Rad Laboratories) on isotropic 14% (wt/vol) acrylamide slab gels (16 by 18 by 0.1 cm or 8 by 6 by 0.1 cm), using the discontinuous buffer system of Laemmli (17). Molecular weight protein standards were Bio-Rad Low or GIBCO-BRL molecular weight standards.

Proteins in gels were detected by a sensitive silver stain (21) or by staining for 30 min with 0.5% Coomassie brilliant blue R-250 in acetic acid-isopropanol-water (1:3:6) and destaining in acetic acid-methanol-water (10:5:85).

Electrophoretic blotting procedures and immunological detection of proteins. Proteins from extracts were first subjected to SDS-PAGE as described above and then transferred to nitrocellulose sheets (0.45 μm; Schleicher & Schuell) in a Trans-Blot cell (Bio-Rad) as previously described (4). Blots were developed with a monospecific anti-ASPND1 (5) or an antimembrane (25) rabbit serum (for detection of the loading control AgMb [antigen from membranes]) at 1/500 dilution as the first antibody. Whenever two proteins were detected in the same sample, the blotted membrane was treated with antibodies to one protein, washed with Tris-buffered saline, blocked for 1 h, and incubated with antibodies against the second protein.

Densitometric quantification of blots. The amount of immunoreactive protein in the bands was quantitated by volume integration, with subtraction of the local background, by using the Bioimage Whole Band Analyzer software (Genomic Solutions, Ann Arbor, Mich.). The values obtained as integrated optical density (IOD) units were used directly for comparison. When needed, the values for ASPND1 bands were corrected for the corresponding value of the loading control (AgMb).

RESULTS

The production of ASPND1 antigen is dependent on the culture medium. Total protein extracts from *A. nidulans* grown in four different culture media (YED, SAB, AMM, and CDA) were analyzed by immunoblotting (Fig. 1) for the presence of the immunodominant antigen ASPND1. An anti-ASPND1 rabbit serum, previously shown to be monospecific (5), confirmed that the characteristic ASPND1 double antigenic band (running around the 50-kDa region) was present at high levels only when the fungus was grown in CDA medium, and this was independent of the temperature of incubation (28 or 37°C). The antigen was detected in all growth phases analyzed (exponential, deceleration, or stationary).

To identify proteins synthesized in all media that could be used as positive controls for constitutive expression, the same blots were reprobed with a rabbit antiserum raised by injecting *A. nidulans* total membranes (25). Under these circumstances, several reactive bands were consistently present in all extracts analyzed. One of these bands, labeled in Fig. 1 as AgMb (running at about 33 kDa), was selected as the internal control for protein loading in further experiments.

Zn^{2+} starvation induces ASPND1 production. To determine which components of CDA medium were responsible for the production of ASPND1, we made a detailed comparison of the two defined media, CDA and AMM, and studied the effects of all of their different components (both quantitatively and qualitatively) on the presence or absence of the antigen. The type of sugar used as the carbon source in CDA medium did not influence ASPND1 production since the amount of antigen was roughly the same regardless of the sugar (Fig. 2A). AS-
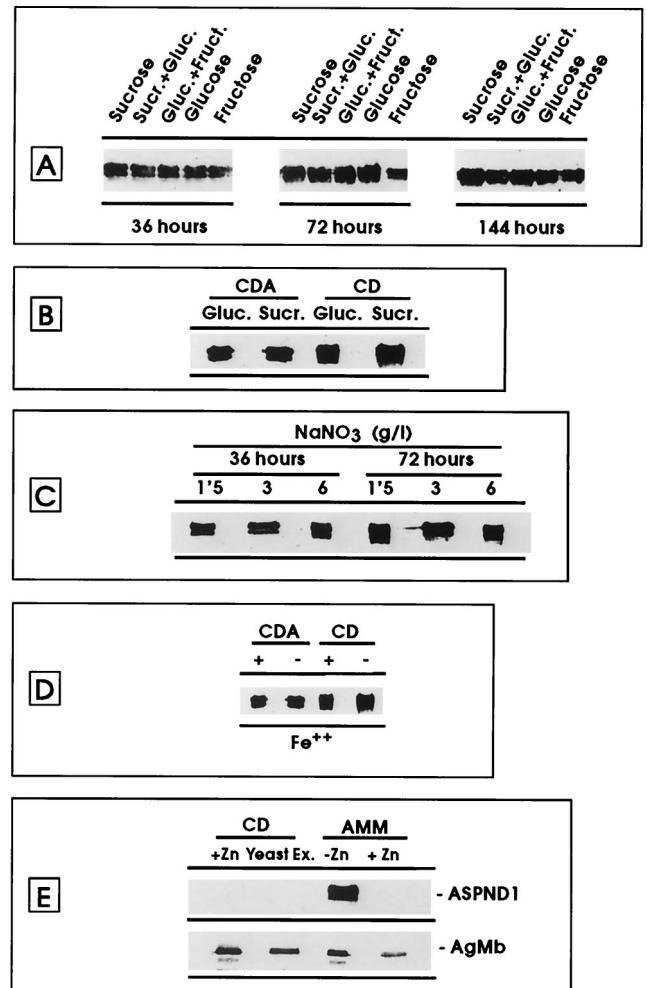

FIG. 2. Immunoblots of total mycelial extracts (20 μg of protein/lane) from *A. nidulans* cultured in CD medium (with the variations and during the times indicated), separated on an SDS–14% polyacrylamide gel, and developed by using as the first antibody a monospecific anti-ASPND1 (panels A to D and ASPND1 in panel E) or an antimembrane (AgMb in panel E) rabbit serum at 1/500 dilution. Experimental details are described in the text.

PND1 was undetectable when grown in AMM, also regardless of the carbon source (data not shown). When the AOAC amino acid mixture was omitted from CDA medium to give the CD medium, ASPND1 production was unaffected, also independently of the carbon source (Fig. 2B). Differences in the levels of nitrate (analyzed in the absence of AOAC amino acid mixture with either glucose or sucrose as the carbon source) did not have any effect on ASPND1 production (Fig. 2C). The absence of Fe^{2+} in both CDA and CD media did not influence the production of ASPND1 (Fig. 2D). However, addition of Zn^{2+} to CD medium abolished ASPND1 production; conversely, when Zn^{2+} was omitted in the preparation of AMM medium, high levels of ASPND1 were detected on the immunoblots (Fig. 2E, line ASPND1). Again, AgMb was used as a constitutive control (Fig. 2E) and did not significantly change. Yeast extract was also inhibitory when added to CD (Fig. 2E) or CDA (not shown) medium, probably because of its undetermined Zn^{2+} content.

Kinetics of ASPND1 production. ASPND1 seemed to be produced continuously during growth in CDA (Fig. 1). How-
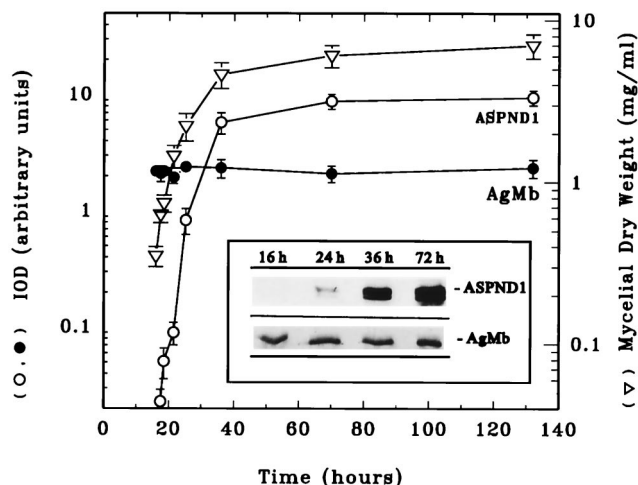
FIG. 3. Kinetics of ASPND1 and AgMb production with time. *A. nidulans* (10^5 conidia/ml) was cultured at 28°C in CD medium. At the indicated times, samples were withdrawn to determine mycelial dry weight (triangles) and to obtain total mycelial extracts, which were separated on an SDS–14% polyacryl-amide gel (20 μg of protein per lane) and immunoblotted by using as the first antibody anti-ASPND1 (insert, ASPND1) or antimembrane (insert, AgMb) rab-bit serum at 1/500 dilution. ASPND1 and AgMb bands were quantified by densitometry, and the IOD values obtained were plotted (on a semilogarithmic scale) against time. AgMb was constitutively produced (black circles), whereas the synthesis of ASPND1 (white circles) increased with time in parallel with the increase in mycelial dry weight (white triangles). Results are means (± standard deviations) of three separate determinations.
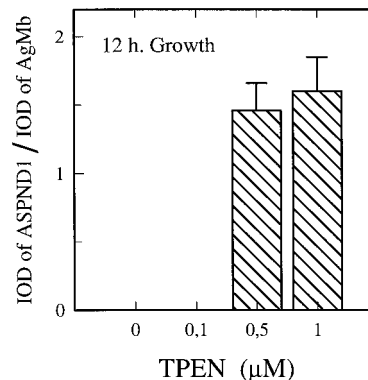

FIG. 4. Effect of zinc chelation on ASPND1 production. *A. nidulans* was cultured for 12 h at 28°C by inoculation of 10^6 conidia/ml in CD medium supplemented with 0.1, 0.5, and 1.0 μm TPEN (a zinc-specific chelator). At the end of growth, total mycelial extracts were separated on an SDS–14% polyacryl-amide gel (20 μg of protein per lane) and immunoblotted with anti-ASPND1 and anti-membrane rabbit serum as the first antibody. Results are means (± standard deviations) of three separate determinations and are expressed as the IOD ratio between ASPND1 and AgMb.

ever, in these early experiments the first samples had been withdrawn after 24 h at 37°C or 39 h at 28°C. To examine earlier time points, cultures were inoculated with 10^5 conidia/ml and grown in CD medium (the simplest producing medium) at 28°C. ASPND1 was not evident on immunoblots until 24 h of growth at 28°C, and its concentration increased exponentially with the increase in mycelial dry weight (Fig. 3), indicating that the antigen was not constitutively produced at early time points. When CDA medium was used, the results were analogous, but ASPND1 was not detectable until around 36 h at 28°C. AgMb used as a loading control was detected at the same level throughout the growth period in all of the media and can therefore be used as a true constitutively expressed protein.

The lack of ASPND1 in the early hours of growth may be due to an inhibitor, such as Zn^{2+}, present in CD medium. To test this hypothesis, we used the specific chelator tetrakis-(2-pyrydylmethyl)ethylenediamine (TPEN) (8). If Zn^{2+} is the inhibitor, then lowering the free Zn^{2+} should enhance ASPND1 production at early time points. Figure 4 shows that this is indeed the case. Concentrations of 0.5 to 1 μM chelator were enough to allow high levels of ASPND1 production in the previously nonproducing 12-h cultures (10^6 conidia/ml), whereas the biosynthesis of the loading control AgMb was not affected by TPEN (not shown). These results are consistent with the idea that trace amounts of Zn^{2+} in the culture me-dium were responsible for the delay in the onset of ASPND1 production. Concentrations of TPEN between 5 to 100 μM prevented conidium germination and were not tested. The effects of TPEN were specifically reversed by the addition of Zn^{2+} (data not shown), and it therefore follows that the onset of ASPND1 production is dependent on the concentration of available Zn^{2+}.

Addition of Zn^{2+} specifically inhibits ASPND1 production but stimulates mycelial growth. The extent of inhibition of ASPND1 biosynthesis was dependent on the amount of Zn^{2+} added to the culture medium (Fig. 5). To prevent any inter-ference in conidium germination the cultures (10^6 conidia/ml) were allowed to grow for 12 h at 28°C. At this point they were supplemented with increasing concentrations of Zn^{2+} (0 to 10 μM); after a further 12 or 36 h of incubation, the amounts of ASPND1 and AgMb were determined by immunoblotting and densitometry (the results were identical at 12 and 36 h, and only the first are shown in Fig. 5A). Concentrations as low as 5 μM Zn^{2+} reduced ASPND1 levels by more than 50%, and a total absence of the antigen was observed with concentrations above 20 μM (data not shown). When the cation was added from the start of culture, 5 μM Zn^{2+} was sufficient to fully prevent ASPND1 production (data not shown). Mycelial growth (measured as total protein in cell extracts) increased with the addition of Zn^{2+} to the medium. Concentrations of 1 μM and higher almost doubled the amount of mycelia with respect to the control without added Zn^{2+}, demonstrating that Zn^{2+} is limiting for growth in CD medium (Fig. 5C).

To determine whether other divalent metal ions had similar effects on the production of ASPND1 protein, we treated cul-tures with 50 μM Co^{2+}, Ni^{2+}, Cu^{2+}, or Ca^{2+}. These metal ions did not inhibit ASPND1 production (Fig. 5B). Zn^{2+} and Cd^{2+} totally prevented ASPND1 biosynthesis, and Mn^{2+} afforded a 40% inhibitory effect. Mycelial growth was maximal in the presence of Zn^{2+} (Fig. 5D). At these levels, Mn^{2+}, Co^{2+}, Ni^{2+}, and Cu^{2+} did not affect significantly growth. Cd^{2+} and Ca^{2+} inhibited growth by about 30%. The biosynthesis of the loading control AgMb was not affected by any of the metals (not shown).

The ASPF2 allergen from *A. fumigatus* is another zinc-re-sponsive protein. Due to the close homology of ASPND1 with the immunodominant allergen ASPF2 from *A. fumigatus*, and since this species is the main etiological agent of the different forms of aspergillosis, we were interested in knowing whether the zinc-dependent response observed in *A. nidulans* also oc-curred in *A. fumigatus* or other *Aspergillus* species. When two different *A. fumigatus* strains, *A. fumigatus* C and *A. fumigatus* R, were grown 48 h in CD medium with or without 50 μM Zn^{2+}, a double band (of faster electrophoretic mobility than

ASPND1 production

(A)

(B)

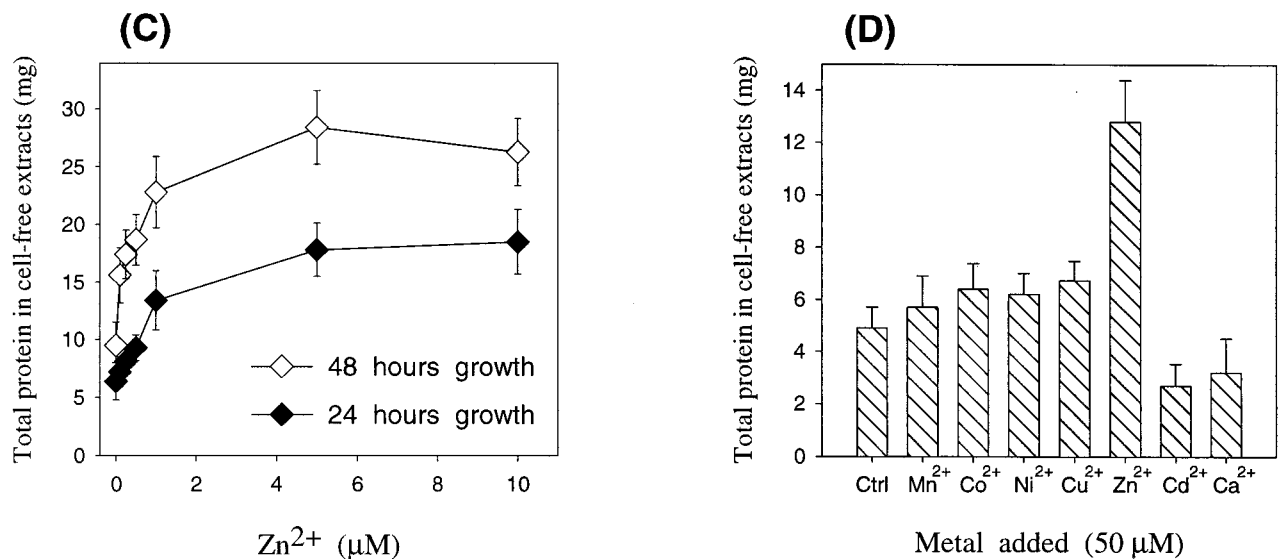## Mycelial growth

(C)

(D)
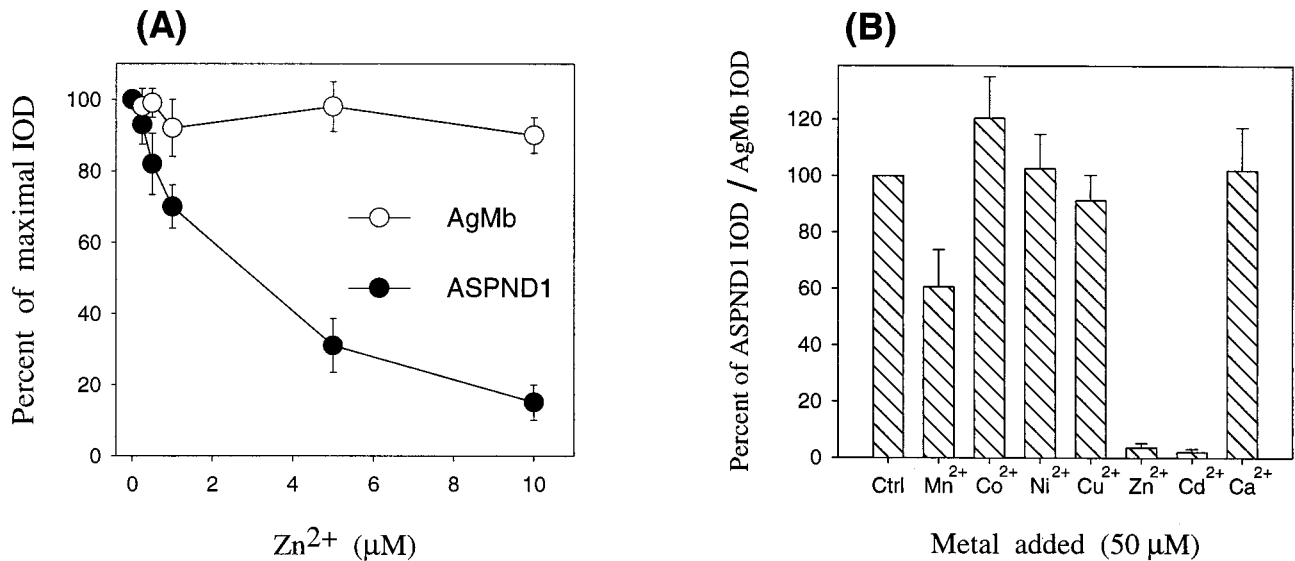
FIG. 5. Effect of the addition of increasing concentrations of Zn^{2+} or of 50 μM divalent ions on ASPND1 production. Several parallel cultures of *A. nidulans* (10^6 conidia/ml) were grown at 28°C for 12 h. Each of the cultures was then supplemented with $ZnSO_4$ to obtain final concentrations of 0, 0.1, 0.5, 1.0, 5.0, and 10.0 μM Zn^{2+} (A and C) or with the indicated divalent metal salts (sulfate form) to achieve a final concentration of 50 μM (B and D). The amount of immunoreactive ASPND1 or AgMb present in total mycelial extracts obtained from all cultures after a further 12 h of growth was determined as described in the text by densitometry of the corresponding immunoblots (A and B). Results in panel A are expressed as the percentage of the maximal IOD measured for ASPND1 or AgMb; results in panel B are expressed as the percentage of the maximal IOD ratio between ASPND1 and AgMb. The amount of total protein in cell extracts (C and D) was determined as indicated in Materials and Methods. Results are means (± standard deviations) of three separate determinations. Ctrl, control.

ASPND1 and corresponding to the 40- and 37-kDa components of ASPF2) was detected in extracts from both strains only when Zn^{2+} was absent from the growth medium (Fig. 6). A relative of the ASPND1 family was also expressed in *A. terreus* and *A. flavus* only in low-zinc culture medium. Immunoblots developed with anti-ASPND1 (Fig. 6) and anti-ASPF2 rabbit monospecific antibodies (data not shown) yielded identical results. The absence of zinc did not dramatically modify the protein patterns, except for ASPND1 and its related antigens (Fig. 7). The patterns of *A. terreus*, *A. nidulans*, and *A.*

flavus were very similar, and only minor differences were observed for both strains of *A. fumigatus*.

DISCUSSION

The influence of the culture medium on the biosynthesis of *Aspergillus* antigens was first described many years ago (14, 32) and has been confirmed on different occasions (4, 13, 15). For instance, antigens harboring proteolytic activities are mainly synthesized in response to low nitrogen or carbon contents in
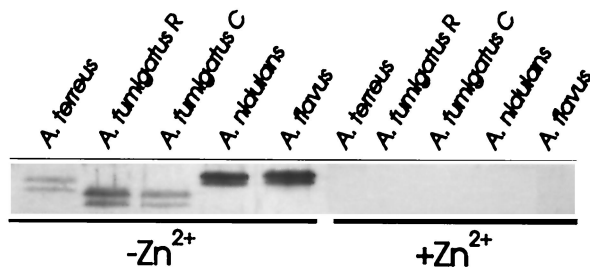
FIG. 6. Presence of zinc-regulated proteins in other *Aspergillus* species. Immunoblots of total mycelial extracts (20 μg of protein/lane) from the indicated *Aspergillus* spp. (10^5 conidia/ml) cultured for 48 h at 28°C in CD medium with or without 50 μM Zn^{2+}, separated on an SDS–14% polyacrylamide gel, and developed by using as the first antibody a monospecific anti-ASPND1 rabbit serum at 1/500 dilution. Experimental details are described in the text.

the culture medium (29) or can be induced by the presence of specific substrates such as elastin (26–28) or collagen (30), both of which are susceptible to proteolytic degradation. Apart from these cases, no other defined inducing signals have been reported. However, the production of some immunodominant antigens is dependent on a particular culture medium; e.g., the closely related *A. fumigatus* ASPF2 and *A. nidulans* ASPND1 antigens were almost exclusively produced in CDA medium (4, 15). Information about the particular components of the media responsible for induction is lacking. To obtain insight into the in vivo signals that direct the fungus to produce a particular protein and into the possible function of that protein as a virulence determinant, we decided to carefully analyze which factors determined the onset of the synthesis of one of these antigens, ASPND1. Here we have shown that ASPND1 synthesis does not depend on the type or amount of the carbon or
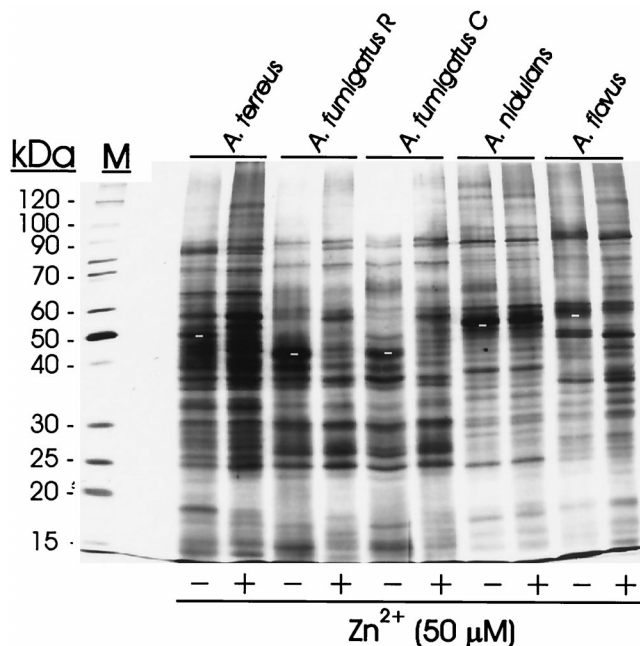
nitrogen source but instead is strongly inhibited by micromolar concentrations of Zn^{2+} or Cd^{2+} in the culture medium. This regulatory system is not exclusive to the *A. nidulans* strain used for the study since the same response was found in other strains tested (data not shown). Furthermore, the response seems to be characteristic of this group of fungi since related species such as *A. fumigatus* (the main pathogenic agent), *A. flavus*, and *A. terreus* also synthesize ASPND1-related antigens in response to zinc starvation.

In trace quantities, zinc is known to be a micronutrient essential to fungal growth, and several *Aspergillus* species have been reported to grow poorly in zinc-deficient cultures (12). Zinc is a functional component of a variety of transcription factors and fungal metalloenzymes ranging from those involved in intermediary metabolism to those involved in the synthesis of nucleic acids. Omission of this cation from the culture medium may have widespread physiological consequences such as a decrease in DNA, RNA, and protein synthesis or a decrease in the number of mitochondria (12). Despite this, very little is known about the molecular mechanisms that cells use to obtain zinc. So far, only two fungal transporter genes, *ZRT1* and *ZRT2* from *Saccharomyces cerevisiae* (34, 35), have been isolated. These two genes define two separate systems: one system (corresponding to *ZRT1*) shows a strong affinity for zinc, and its activity markedly increases in zinc-deprived cells (34). The second system (corresponding to *ZRT2*) has a lower affinity for zinc and is not regulated by zinc availability (35).

The human body has developed a metal-withholding defense system in which metal-binding proteins restrict access of microbial invaders to the host's metals (2). Iron deprivation induces the synthesis of siderophores and several bacterial outer membrane proteins involved in iron acquisition (18). These iron-repressible outer membrane proteins are found in many bacterial pathogens both in vitro and in vivo (22, 33), and there is increasing evidence that these proteins are important virulence determinants (10, 23, 31). Siderophore production induced by low iron concentrations has also been found in *Aspergillus*, although the direct involvement of iron in pathogenesis has not yet been demonstrated (3).

The metal content of lung tissues has never been correlated with the expression of *Aspergillus* virulence factors. It would be interesting to determine the environmental signals that are responsible for enhancing virulence in the lung. Zn^{2+} levels in human plasma and tissues may vary with aging and in several pathological states, but in any case, physiological levels of free zinc are below 20 nM (9, 20). Zinc limitation (independently or together with iron limitation) could indicate to the fungus that it is within a living mammal and function as a switch to induce the synthesis of proteins required to acquire and transport zinc, ensuring that these proteins are synthesized only when needed. ASPND1 and ASPF2 may be the first reported members of a family of zinc-responsive proteins, apparently widely distributed among the aspergilli. Whereas these proteins are only expressed in vitro under conditions of Zn^{2+} starvation, they are readily synthesized in vivo, as demonstrated by the existence of high levels of circulating antibodies specifically directed against them in several forms of aspergillosis (ABPA and aspergilloma) (1, 4, 7).

To our knowledge, this is the first description of zinc-regulated proteins in *Aspergillus*. Negative regulation by zinc in eukaryotes has been reported for at least two other cases. The most similar is represented by the above-mentioned *S. cerevisiae ZRT1* gene (the first gene of this class described in any organism), whose expression was induced at the transcriptional level by zinc limitation (34). In a human cell culture system,

FIG. 7. Protein patterns of different *Aspergillus* species in presence or absence of zinc. Silver-stained SDS–14% polyacrylamide gel profiles of total mycelial extracts (5 μg of protein/lane) from the indicated *Aspergillus* spp. (10^5 conidia/ml inoculum) cultured for 48 h at 28°C in CD medium with or without 50 μM Zn^{2+}. ASPND1 and related antigens are indicated in − Zn^{2+} lanes by white dashes. M, molecular mass markers.

addition of Zn^{2+} to the medium inhibited the induction of ornithine decarboxylase (ODC) activity in ODC-overproducing L1210-DFMO cells. The decrease in activity was accompanied by a proportional decrease in the content of immunoreactive ODC protein, whereas the level of ODC mRNA was not affected significantly (11). Preliminary experiments suggest that in the case of ASPND1, major regulation takes place at the transcriptional level. Confirmation of these data and identification and characterization of putative zinc-responsive elements probably present in the 5′ regulatory region of the *A. nidulans* ASPND1 gene are currently been addressed at our laboratory.

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