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Steroid Hormone Regulation of the *Achlya ambisexualis* 85-Kilodalton Heat Shock Protein, a Component of the *Achlya* Steroid Receptor Complex

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The steroid hormone antheridiol regulates sexual development in the fungus *Achlya ambisexualis*. Analyses of in vivo-labeled proteins from hormone-treated cells revealed that one of the characteristic antheridiol-induced proteins appeared to be very similar to the *Achlya* 85-kilodalton (kDa) heat shock protein. Analysis of in vitro translation products of RNA isolated from control, heat-shocked, or hormone-treated cells demonstrated an increased accumulation of mRNA encoding a similar 85-kDa protein in both the heat-shocked and hormone-treated cells. Northern (RNA) blot analyses with a *Drosophila melanogaster* hsp83 probe indicated that a mRNA species of approximately 2.8 kilobases was substantially enriched in both heat-shocked and hormone-treated cells. The monoclonal antibody AC88, which recognizes the non-hormone-binding component of the *Achlya* steroid receptor, cross-reacted with *Achlya* hsp85 in cytosols from heat-shocked cells. This monoclonal antibody also recognized both the hormone-induced and heat shock-induced 85-kDa in vitro translation products. Taken together, these data suggest that similar or identical 85-kDa proteins are independently regulated by the steroid hormone antheridiol and by heat shock and that this protein is part of the *Achlya* steroid receptor complex. Our results demonstrate that the association of hsp90 family proteins with steroid receptors observed in mammals and birds extends also to the eucaryotic microbes and suggest that this association may have evolved early in steroid-responsive systems.

The water mold *Achlya ambisexualis* is a eucaryotic, filamentous fungus in which sexual reproduction is regulated by well-characterized steroid hormones (4, 22, 54). There are at least two mating types in *A. ambisexualis*, and these are usually referred to as male and female. Each of these mating types produces a specific fungal steroid hormone which is secreted into the medium and which mediates both chemotropic and developmental changes in cells of the opposite mating type. In this respect, the *Achlya* steroids act as both pheromones and hormones.

The fungal steroid hormone antheridiol, normally produced by female strains of *A. ambisexualis*, has been isolated (30) and characterized (2). Studies in our laboratory have focused on the early developmental events which occur when antheridiol is added to vegetatively growing cultures of the male mating type, strain E87 (9–11, 48). In E87, one of the earliest developmental events seen at the morphological level after treatment of cells with the hormone is the formation of distinct structures called antheridial branches (3). Both the time of onset of branching and the percent of hyphae branched are directly related to the amount of hormone added (9, 30). Thus, the response is very synchronous, and a high percentage of the hyphae is target tissue for the hormone. For these reasons, *A. ambisexualis* represents an interesting system in which to investigate both the mechanism of action of steroid hormones and the evolution of steroid hormone systems.

In previous studies (9–11), we identified a number of characteristic *Achlya* proteins which appear to be regulated by antheridiol. These proteins could be localized to specific cell fractions (9–11). The hormone also appears to induce changes in the synthesis or processing of certain *Achlya*-secreted glycoproteins (9). One of the prominent antheridiol-induced proteins exhibits a molecular weight of 85,000 and was found both in the nuclear and cytoplasmic fractions of antheridiol-treated cells (10). This protein seemed very similar in electrophoretic behavior to the *Achlya* 85-kilodalton (kDa) heat shock protein (49), which is one of the several characteristic heat shock proteins observed in *A. ambisexualis* (33, 34, 49). We have therefore investigated the relationship between these two 85-kDa proteins and their regulation by both heat shock and by the steroid hormone antheridiol.

In *A. ambisexualis*, the response to antheridiol and the response to heat shock are not synonymous at either the morphological or the biochemical levels (9–11, 33, 34, 48, 49). Nevertheless, we report here that both hormone treatment and heat shock induce the accumulation of translatable mRNA encoding similar 85-kDa proteins. The 85-kDa *Achlya* heat shock- and steroid hormone-regulated proteins are antigenically related to one another and to the non-hormone-binding component of the putative receptor for the *Achlya* steroid hormone antheridiol (38). Our results indicate that the association of hsp90 family proteins with steroid receptors observed in mammals and birds (15, 24, 37, 42–45; reviewed in reference 35) extends also to the eucaryotic microbes and suggest that this association may have evolved quite early in steroid-responsive systems.

MATERIALS AND METHODS

Heat shock treatment and in vivo labeling conditions. Cells of *A. ambisexualis* J. Raper strain E87 were grown as described previously (49). Control cells were maintained at 28°C and were labeled for 40 min with 3.0 μCi of [^{35}S]methionine per ml (>1,000 Ci/mmol; Dupont, NEN Research

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Products). Heat-shocked cells were subjected to a rapid temperature elevation from 28 to 37°C and were labeled as described for controls.

Hormone treatment and in vivo labeling conditions. Cells were grown as described previously (9–11). Antheridiol (a gift from T. C. McMorris) was added to the cultures to a final concentration of 1 ng/ml (2.1 nM) in 0.01% methanol. Controls received 0.01% methanol only. Cells were labeled with 3.3 μCi of [^{35}S]methionine per ml for 70 min at 20 or 90 min after addition of hormone or methanol or both.

Cell fractionation and preparation of cytoplasmic and nuclear proteins. The cells were collected by filtration through Miracloth (Johnson and Johnson, Chicopee Division, New Brunswick, N.J.), washed, and broken open in a Willems polytron homogenizer (Brinkmann Instruments) as previously described (47). Cell homogenates were filtered through Miracloth, and the crude filtrate was treated as described by Brunt and Silver (10) to obtain the cytoplasmic and crude nuclear pellet. Total nuclear proteins were prepared from the further-purified nuclear fraction as previously described (10, 49).

Preparation of cytosolic proteins and immunoaffinity isolation using AC88. The postnuclear supernatant was obtained as previously described (10, 49). The cytosolic fraction was obtained by centrifugation of the postnuclear supernatant at 100,000 × *g* for 60 min. Cytosolic proteins were concentrated by ammonium sulfate precipitation and were suspended in 10 mM Tris hydrochloride, pH 8, containing 10% (vol/vol) glycerol. The cytosols from control and heat-shocked cultures were incubated in the presence of the monoclonal antibody AC88 (38) coupled to Affigel 10 (Bio-Rad Laboratories). The gel was collected by centrifugation (2,300 × *g* for 5 min); it was washed, and the proteins were eluted from Affigel 10 with 4 M NaSCN and precipitated with 10% (wt/vol) trichloroacetic acid as described by Riehl et al. (38). The precipitated proteins were then solubilized in 125 mM Tris hydrochloride (pH 6.8)–5% (vol/vol) 2-mercaptoethanol–1% (wt/vol) sodium dodecyl sulfate (SDS)–10% (vol/vol) glycerol by heating at 100°C for 5 min in preparation for one-dimensional SDS-polyacrylamide slab gel electrophoresis.

Isolation of total cellular RNA. Total RNA was isolated from cells grown at 28°C (controls), cells heat shocked at 37°C for 30 min, and cells treated with the steroid hormone antheridiol for 90 min at a final concentration of 1 ng/ml by using a modification of the method of Weiben (55). Mycelia were harvested, washed, frozen in liquid nitrogen, and ground to a powder. To the frozen powder, 8.5 ml of a 6 M guanidine HCl–20 mM sodium acetate (pH 5.1)–10 mM dithiothreitol buffer was added per gram of wet weight of mycelium, and the mixture was vortexed for 80 s in a sterile plastic centrifuge tube. The brei was spun at 17,000 × *g* for 20 min at 4°C. The resultant supernatant was layered over a 3-ml cushion of 5.7 M cesium chloride containing 0.1 M EDTA and 30 mM sodium acetate (pH 5.1) and spun at 87,000 × *g* for 22 h at 22°C in a swinging bucket rotor. The RNA pellet was suspended in sterile, twice-distilled H_2O and concentrated by precipitation. The RNA was suspended in sterile water and stored at −70°C.

In vitro translation of *Achlya* RNA and immunoprecipitation with AC88. Equivalent amounts of total RNA (10 μg for isoelectric focusing [IEF] two-dimensional gel analysis and 20 μg for immunoprecipitation studies) from control, heat-shocked, and hormone-treated cells were translated in vitro in a rabbit reticulocyte lysate system (Bethesda Research Laboratories, Inc.) by using [^{35}S]methionine to label the translation products. For the immunoprecipitation studies, protein samples containing equal amounts of acid-precipitable radioactivity were adjusted to 1 ml with 10 mM Tris hydrochloride, pH 8.0, containing 10% glycerol. Each sample was preadsorbed for 1 h at 21°C with a nonspecific heterologous monoclonal antibody anti-gp50 (a gift from T. Palladino and J. Gurd) and protein A-Sepharose. The protein A-Sepharose-protein complexes were removed by centrifugation. To the supernatants, 62 μg of AC88, a monoclonal antibody raised against an 88-kDa non-hormone-binding protein from the putative *Achlya* steroid hormone receptor complex (38) was added, and the samples were incubated for 1 h at 21°C. Subsequently, the antibody-protein complexes were adsorbed to a 1:4 (wt/vol) suspension of protein A-Sepharose CL-4B (Sigma Chemical Co.) in 25 mM KPO_4, pH 7.2, buffer containing 10% (vol/vol) glycerol with 0.02% NaN_3 added, for 1 h at 21°C. The gel was collected and washed with a 25 mM KPO_4 buffer containing 300 mM KCl and 0.2% Nonidet P-40. The proteins were eluted from protein A-Sepharose with 4 M NaSCN, precipitated with 20% trichloroacetic acid (wt/vol), and washed with 95% ethyl alcohol, followed by acetone. The protein samples were prepared for one- and two-dimensional gel electrophoresis as described by Brunt and Silver (10).

Electrophoretic procedures. The in vivo ^{35}S-labeled proteins and ^{35}S-labeled in vitro translation products were analyzed by using the two-dimensional gel system of O'Farrell (32) in which the first-dimension gels are run in the basic to acidic direction. The second dimension consisted of an 8 to 15% SDS-polyacrylamide gel with the discontinuous buffer system of Laemmli (26). The first-dimension IEF gels were loaded with protein samples containing equal amounts of radioactivity. The ^{35}S-methionine-labeled proteins were located by fluorography (28).

The immunoaffinity-isolated cytosolic proteins were analyzed on one-dimensional SDS-polyacrylamide slab gels by using a 7.5% polyacrylamide gel and the buffer system of Laemmli (26). The gels were stained with 0.05% (wt/vol) Coomassie brillant blue R250 to visualize the proteins.

The immunoaffinity-isolated ^{35}S-labeled in vitro translation products were separated on an 8 to 15% SDS-polyacrylamide gradient gel and visualized by fluorography (28).

RNA and DNA blot hybridizations. Restriction enzyme-digested genomic DNA was separated on a 1% agarose gel and transferred to nitrocellulose by capillary action. Southern hybridizations were carried out under moderate stringency conditions (30 to 40% [vol/vol] formamide–0.75 M NaCl–0.075 M sodium citrate at 37°C). Final washes were with 15 mM NaCl–1.5 mM sodium citrate–0.1% SDS at 50°C for 1 h.

RNA was separated in a formaldehyde–1.5% agarose gel system and transferred to nitrocellulose. Northern hybridizations were carried out at 50% (vol/vol) formamide–0.75 M NaCl–0.075 M sodium citrate at 42°C. Final washes were as described for Southern hybridizations.

The hsp83 probes used were coding sequences from both pPW244, a genomic clone from *Drosophila melanogaster* Oregon R (21), and from subclone 6.1, a genomic sequence from *D. melanogaster* Canton S (20). The *Saccharomyces cerevisiae* actin gene clone pYF211 (31) was used in the Northern analysis to ensure equal loading of RNA. Probes were labeled with [α-^{32}P]CTP by using the random-primer method of Feinberg and Vogelstein (17). DNA probes were used for both DNA and RNA blot hybridizations.

Northern blots hybridized sequentially with the *Drosophila* hsp83 and the yeast actin probes were scanned by using

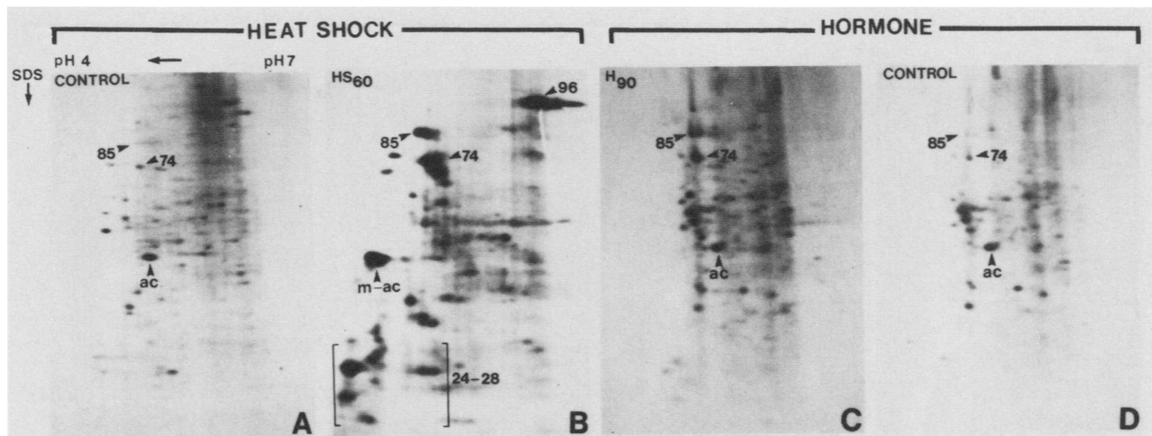

FIG. 1. Comparison of [^{35}S]methionine-labeled nuclear proteins from control, heat-shocked, and hormone-treated cells. For the hormone studies, cells growing at 28°C were treated with antheridiol for 20 or 90 min and then were labeled for 70 min with [^{35}S]methionine in the continued presence of hormone. Since antheridiol is delivered in 0.01% methanol, control cells for the hormone studies were treated with this concentration of methanol and labeled as described for the hormone-treated cells. For heat shock, cells growing at 28°C were shifted to 37°C for 20 min and then were labeled with [^{35}S]methionine for 40 min at 37°C. Control cells for the heat shock studies were maintained at 28°C and labeled as for heat-shocked cells. Nuclear proteins were isolated and analyzed on IEF-SDS two-dimensional gels. The pH range of the first dimension IEF gels and the direction of migration are as indicated in panel A. Shown are fluorograms of ^{35}S-labeled nuclear proteins from control cells (A), heat-shocked cells (B), antheridiol-treated cells (C), and methanol-treated cells (i.e., the control for the hormone studies) (D). Specific proteins are indicated by their molecular weights. Ac, Actin; m-ac, modified actin.

a Bio-Rad model 1650 densitometer. The scans of the hybridization signals obtained on the actin blots were used to adjust the signal detected with the *Drosophila* hsp83 probe, such that the difference in intensity of the signal with heat shock or hormone treatment was due solely to an increase in a specific mRNA and not to a difference in total RNA loaded per track.

RESULTS

Similar 85-kDa proteins are induced in *A. ambisexualis* by heat shock and by steroid hormone. In *A. ambisexualis*, the steroid hormone antheridiol has been shown to induce the synthesis and/or accumulation of a number of characteristic proteins (9–11), one of which exhibits a molecular weight of 85,000. This hormone-regulated protein was found in both the cytoplasmic and nuclear fractions of the *Achlya* mycelium (10). As shown in Fig. 1, the electrophoretic behavior of the 85-kilodalton (kDa) hormone-induced protein (Fig. 1C) appeared to be similar to that of the characteristic 85-kDa *Achlya* heat shock protein (Fig. 1B). The [^{35}S]methionine labeling of the 85-kDa hormone-induced protein (Fig. 1C) was not as marked as the increase observed with heat shock (Fig. 1B).

Although 85-kDa proteins are induced by both hormone and heat shock treatments, these responses are not synonymous at either the morphological or the biochemical level (9–11, 33, 34, 48, 49) and specific in vivo-labeled proteins (Fig. 1) and in vitro translation products (see Fig. 3) unique to each response are observed. For example, although some induction of *Achlya* hsp74 is observed with hormone (Armavil, Brunt, and Silver; manuscript in preparation), the synthesis of hsp96 and hsp24 through hsp28, as well as the characteristic posttranslational modification of actin (49), are seen only with heat shock (Fig. 1A through D).

In some cell types, hsp90 family proteins are reported to be exclusively cytoplasmic (13, 27, 51, 56); however, in *A. ambisexualis*, hsp85 is observed in both the nuclear and the cytoplasmic fractions (49). The newly synthesized steroid-induced 85-kDa protein was also observed in both the nuclear (Fig. 1) and cytoplasmic fractions (10) of hormone-treated cells.

Both hormone treatment and heat shock result in the accumulation of translatable mRNA encoding similar 85-kDa proteins. In vivo protein synthesis studies (Fig. 1) indicated that both heat shock and antheridiol induce the synthesis and/or accumulation of 85-kDa proteins. To assess whether this change reflected regulation at the mRNA level, populations of translatable RNA from control, heat-shocked, and hormone-treated cells were compared by in vitro translation in a rabbit reticulocyte lysate system (Fig. 2). Analysis of the translation products on two-dimensional gels suggested that there was an increase in the steady-state levels of mRNA encoding similar 85-kDa proteins in both heat-shocked (Fig. 2B) and hormone-treated (Fig. 2C) cells. This translation product was much reduced in the translatable RNA population from control cells (Fig. 2A and D). Of interest is the streaked appearance on the two-dimensional gels of both the in vitro-translated hsp85 and 85-kDa hormone-regulated proteins. When labeled in vivo, these 85-kDa proteins focus in the IEF dimension as fairly discrete spots at an average pI of 5.4 (Fig. 1). However, although other in vitro translates focus well, the in vitro-translated 85-kDa proteins routinely form a streak across the entire gel (Fig. 2). Treatment of the in vitro translation products with RNase A or alkaline phosphatase did not alter the electrophoretic behavior of the 85-kDa translation products (data not shown). The basis for this unusual electrophoretic behavior is not known at present; however, it appears to be characteristic of the 85-kDa in vitro translation products.

While both heat and hormone treatment resulted in increased levels of mRNA for 85-kDa proteins, there were also changes in in vitro translates unique to each response. For example, a message for a 34-kDa protein was decreased with heat shock (Fig. 2B) but not with hormone (Fig. 2C), while the synthesis or accumulation of message for a 24.3-kDa protein was greatly enhanced with steroid hormone (Fig. 2C) but not with heat shock (Fig. 2B).

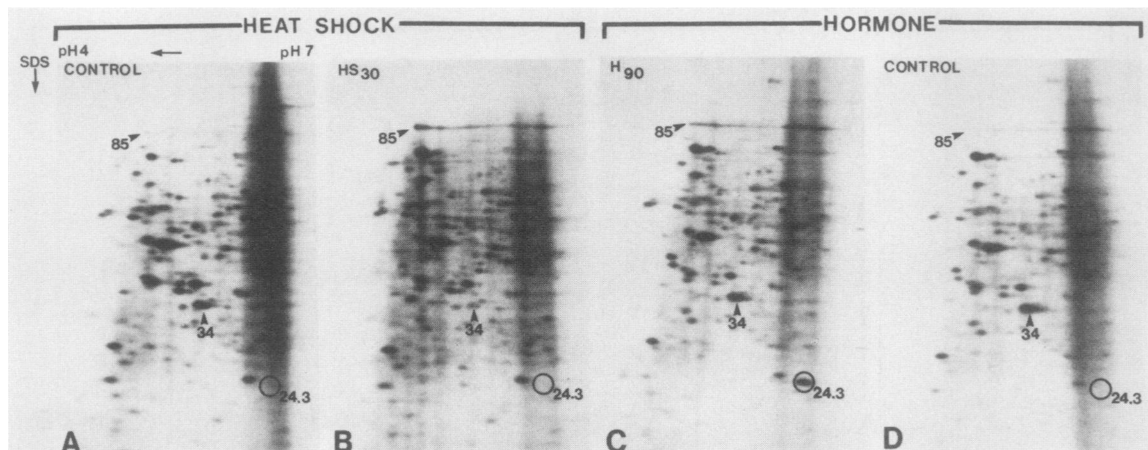

FIG. 2. Analysis of in vitro translation products of RNA from control, heat-shocked, and hormone-treated cells. RNA was isolated from control cells, cells heat shocked for 30 min (HS30), and cells treated with the steroid hormone antheridiol for 90 min (H90). For the hormone studies, control cells were treated with methanol at a final concentration of 0.01% for 90 min. Total RNA was isolated, and 10 μg of each was translated in a reticulocyte lysate system by using [^{35}S]methionine. The in vitro translation products were analyzed on two-dimensional IEF-SDS gels as described in the legend to Fig. 1. Gels were loaded with protein samples containing equal amounts of acid-precipitable radioactivity. ^{35}S-labeled in vitro translation products of RNA from control (A), heat-shocked (B), hormone-treated (C), and methanol-treated (D) cells are shown. Specific proteins are indicated by their molecular weights.

Estimation of the size and level of accumulation of the *Achlya* heat shock and hormone-induced mRNAs encoding the 85-kDa proteins. Northern blot analyses with a heterologous hsp85 probe were used to identify the mRNAs encoding the *Achlya* hsp85 and the 85-kDa hormone-regulated proteins. Total RNA was isolated from control, heat-shocked, and hormone-treated cells; it was separated by formaldehyde-agarose gel electrophoresis and transferred to nitrocellulose for hybridization analysis. Since Southern hybridizations (see below) showed that the *Drosophila* hsp83 gene has significant homology to the putative *Achlya* hsp85 DNA sequence(s), *Drosophila* hsp83 probes (see Materials and Methods) were used in the Northern blot analysis of *Achlya* RNA. As shown in Fig. 3A, RNA complementary to the *Drosophila* hsp83 gene probe was present under all three experimental conditions. However, relative to controls (Fig. 3A, lane 1), heat shock resulted in an approximately three- to fourfold increase (Fig. 3A, lane 2) and steroid hormone treatment resulted in a two- to threefold increase (Fig. 3A, lane 3) in the level of this *Achlya* message. These increased message levels correlate well with the observed increases in amounts of the 85-kDa in vitro translation products (Fig. 2).

To ensure that each lane (Fig. 3A) contained equal amounts of *Achlya* RNA, the same blot was hybridized with a yeast actin probe (Fig. 3B). In vitro translation data (Fig. 2) indicated that the accumulation of mRNA encoding *Achlya* actin was not altered with heat shock or steroid hormone. In contrast to the results obtained with the hsp83 probe, similar levels of actin mRNA were observed under all three conditions.

The size of the *Achlya* heat shock and hormone-induced messages complementary to the *Drosophila* hsp83 probe was approximately 2.8 kilobases. This is consistent with the sizes of hsp90 mRNAs reported for several other organisms, which range from 2.3 to 3.0 kilobases (7, 14, 16, 19).

***Achlya* hsp85 is antigenically related to the non-hormone-binding component of the *Achlya* steroid receptor.** Riehl et al. (38), by using a protocol similar to that used to isolate the 90-kDa non-steroid-binding component of the molybdate-stabilized chick progesterone receptor (50), isolated a similar component of the *Achlya* antheridiol receptor. This *Achlya* protein exhibited a molecular weight of approximately 88,000. A monoclonal antibody, AC88, raised against this protein (38) shows a broad range of cross-reactivity with avian and mammalian 90-kDa non-steroid-binding receptor component proteins (23, 38, 43). Monoclonal AC88 was used to determine whether the *Achlya* 88-kDa receptor component was related to the *Achlya* 85-kDa heat shock protein (49). Cytosol from control and heat-shocked cells was prepared and adsorbed to AC88 which had been covalently

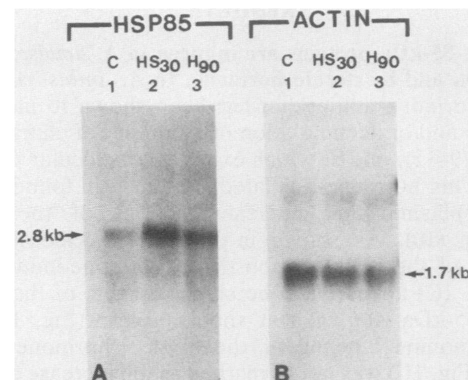

FIG. 3. Northern analysis of RNA from control, heat-shocked, and hormone-treated cells. Total RNA was isolated from control, heat-shocked, and hormone-treated cells. The heat shock and hormone treatment periods were identical to those described in the legend to Fig. 2. The RNA was separated on a 1.5% formaldehyde-agarose gel and transferred to nitrocellulose. Each lane contained 10 μg of total RNA. The DNA probes used were from an hsp83 clone from *D. melanogaster* Canton S and an actin clone from *S. cerevisiae* (see Materials and Methods). Hybridizations were carried out in 50% formamide at 42°C for 20 to 24 h. The final washes of the filters were in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 50°C. The nitrocellulose filter was probed initially with the *Drosophila* hsp83 clone, the radioactivity was then removed, and the filter was reprobed with the actin clone. RNA from control (lane 1), heat-shocked (lane 2), and hormone-treated (lane 3) cells is shown. Molecular sizes (in kilobases) are indicated at the left and right sides.

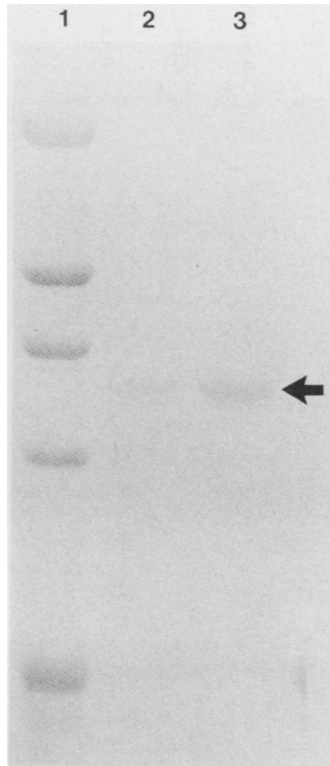

FIG. 4. Immunoadsorption of *Achlya* hsp85 with AC88. Cytosol prepared from control and heat-shocked cells was incubated with the monoclonal antibody AC88 coupled to Affigel 10. Proteins adsorbed to the antibody resin were eluted and subjected to SDS-polyacrylamide gel electrophoresis on a 7.5% polyacrylamide gel. The gel was stained with Coomassie blue to visualize the proteins. The molecular weight standards are (from top to bottom) myosin heavy chain (212,000), β-galactosidase (130,000), phosphorylase B (97,000), bovine serum albumin (68,000), and ovalbumin (43,000). Molecular-weight standards (lane 1), cytosolic proteins from control cells (lane 2), and cytosolic proteins from heat-shocked cells (lane 3) are shown.

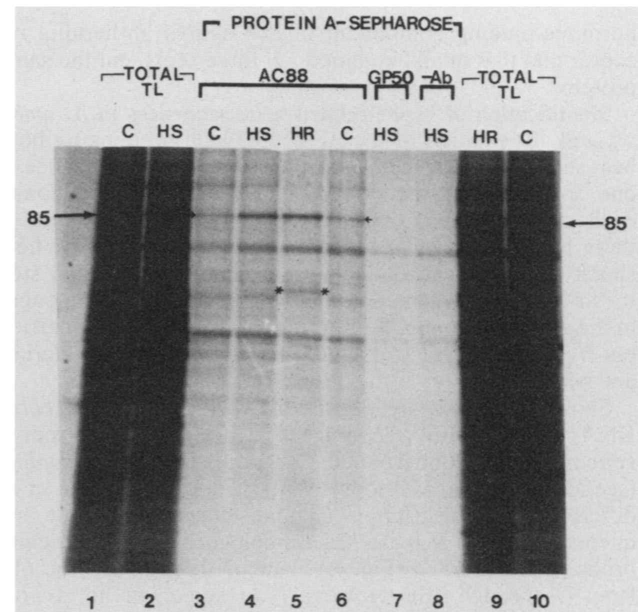

FIG. 5. Analysis of the antigenic relatedness of the in vitro translation products of RNA from heat-shocked and hormone-treated cells to the non-hormone-binding component of the *Achlya* receptor. Total RNA was isolated from control, heat-shocked, and hormone-treated cells. The heat shock and hormone treatment periods were identical to those described in the legend to Fig. 2. The RNA was translated in a rabbit reticulocyte lysate system by using [^{35}S]methionine. The translation products were each first preadsorbed with a heterologous monoclonal antibody, anti-GP50 (specific for a rat brain glycoprotein), and protein A-Sepharose. The nonadsorbed translation products were then incubated with either protein A-Sepharose and AC88 (lanes 3 to 6), protein A-Sepharose and anti-GP50 (lane 7), or protein A-Sepharose alone (lane 8). The proteins were eluted from the protein A-Sepharose and analyzed on 8 to 15% SDS-polyacrylamide gels, and fluorograms were prepared. Shown for comparison in lanes 1, 2, 9, and 10 are the total in vitro translation products of RNA from control (lane 1), heat-shocked (lane 2), hormone-treated (lane 9), and hormone control (lane 10) cells. The position of the 85-kDa translation product is indicated (arrow), as is the 61-kDa protein (*) (see text).

coupled to Affigel 10. SDS gel electrophoresis of the proteins eluted from the antibody resin (Fig. 4) showed that relative to controls (Fig. 4, lane 2), greater amounts of an 85- to 88-kDa protein cross-reacting with AC88 accumulated in heat-shocked cells (Fig. 4, lane 3, arrow). These results indicate that the *Achlya* 85-kDa heat shock protein is antigenically related to the non-hormone-binding component of the putative *Achlya* steroid receptor. This is the first demonstration in a nonvertebrate system of the possible association of an hsp90 family protein with a steroid receptor.

The in vitro-translated heat shock and hormone-induced 85-kDa proteins are antigenically related to the non-hormone-binding component of the antheridiol receptor. Monoclonal antibody AC88 was used further to test the similarity of the in vitro-translated heat shock and hormone-induced 85-kDa proteins to one another and to the non-hormone-binding component of the antheridiol receptor. RNA was prepared from heat-shocked and hormone-treated cells and their appropriate controls and was translated in vitro. Figure 5, lanes 1, 2, 9, and 10, contain the total in vitro translates before precipitation with antibody. As shown in Fig. 5 and also as shown earlier (Fig. 2), both heat shock (Fig. 5, lane 2) and antheridiol treatment (Fig. 5, lane 9) resulted in the increased accumulation of translatable message for an 85-kDa protein. Neither protein A alone (Fig. 5, lane 8) nor protein A plus an unrelated antibody, antigp50 (Fig. 5, lane 7), immunoprecipitated the 85-kDa protein in translation products of heat shock RNA. AC88 did, however, specifically immunoprecipitate the 85-kDa proteins enriched in the translation products from both heat-shocked and hormone-treated cells (Fig. 5, lanes 4 and 5, arrow). Translation products from control cells contained only low levels of immunoprecipitated 85-kDa protein (Fig. 5, lanes 3 and 6). The 85-kDa proteins immunoprecipitated by AC88 had the same streaked appearance on two-dimensional gels (data not shown) as had the 85-kDa proteins observed in the total in vitro translation products (Fig. 2). In addition to the 85-kDa protein, AC88 specifically immunoprecipitated a 61-kDa protein present only in the in vitro translation products of hormone-induced cells (Fig. 5, lane 5, *). The relationship between the 61-kDa protein and the 85-kDa protein is currently under investigation. Additional bands evident in the immunoprecipitates were found to be due to nonspecific binding to protein A-Sepharose (Fig. 5, lane 8). The results described above indicate that both heat shock and steroid hormone treatment result in an increase in the steady-state levels of mRNA encoding 85-kDa proteins which are antigenically related to one another and to the putative non-

hormone-binding component of the *Achlya* antheridiol receptor and that in all likelihood, all three represent the same protein.

Identification of hsp85-related gene sequences in *A. ambisexualis*. Regulation of the *Achlya* 85-kDa proteins by both heat shock and steroid hormone might reflect the presence of one or more hsp85 gene sequences in the *Achlya* genome, each of which is controlled by both types of effectors, or there might be specific hsp85 sequences regulated by heat shock and others regulated by hormone. As an initial step towards identifying the potential number of hsp85 sequences in the *Achlya* genome, Southern hybridizations were carried out by using cloned *Drosophila* hsp83 genes (see Materials and Methods).

Shown in Fig. 6 are Southern hybridizations of *Achlya* DNA probed with a coding sequence fragment from a genomic hsp83 clone from the Oregon R strain of *Drosophila* (see Materials and Methods). With this probe, in 7 out of 10 different single-restriction enzyme digests, a single sequence, which is at least 70% homologous to this particular probe, was observed (Fig. 6A, lanes 7 through 14; Fig. 6B, lanes 9 through 16). However, on screening an *Achlya* genomic library with this *Drosophila* probe, two distinct *Achlya* hsp85 sequences were isolated (12). The expression patterns of these distinct *Achlya* sequences are currently being investigated.

DISCUSSION

In the filamentous fungus *A. ambisexualis* development of sexual stages (gametangia) and subsequent events in mating are regulated by steroid hormones (reviewed in references 3, 4, 22, and 54). One of the *Achlya* mating hormones is antheridiol, a C29 sterol (3). Many of the biochemical responses of *A. ambisexualis* to the steroid hormone antheridiol are analogous to those which have been characterized in steroid-responsive vertebrate systems. For example, previous early studies (48, 53) have shown that as is true in certain vertebrate systems (reviewed in reference 1), both total poly(A^+) RNA synthesis and protein synthesis increase after the addition of antheridiol to cultures of the male strain of *A. ambisexualis*. These biochemical changes are required for the induction and differentiation of the *Achlya* male gametangia (antheridia).

Recently an antheridiol-binding protein complex was identified in the cytosol of the male strain, E87, but not in the female strain. This 192,000 molecular weight protein complex was shown to have biochemical properties similar to those of steroid receptors in higher organisms (39–41). For example, as is true for vertebrate steroid receptors, the molybdate-stabilized *Achlya* steroid receptor complex was shown to have a sedimentation coefficient of 8*s*, and this could be converted to 4*s* by salt treatment (41).

One prominent antheridiol-regulated protein which we identified previously is an acidic 85-kDa protein (10). An acidic 85-kDa protein is also one of the prominent *Achlya* heat shock proteins (49). A comparison of the proteins from heat-shocked and hormone-treated cells on two-dimensional gel analyses revealed that the electrophoretic behaviors of the *Achlya* 85-kDa heat shock and hormone-induced proteins were identical. With either heat shock or steroid hormone treatment, at least some proportion of the newly synthesized 85-kDa protein, as judged by [^{35}S]methionine labeling, is found in the nuclear fraction of the mycelium (Fig. 1). Some minor but reproducible differences in localization were noted, however. For example, in heat-shocked

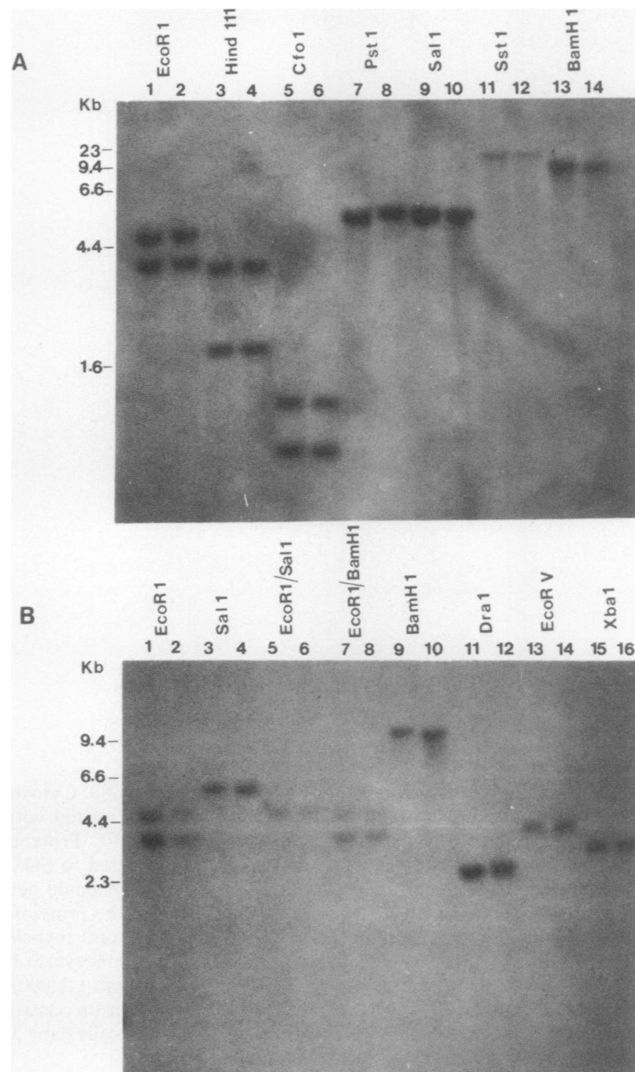

FIG. 6. Southern analyses of *Achlya* genomic DNA. *Achlya* genomic DNA was digested with the restriction enzymes indicated on the figure. The DNA was separated on a 1% agarose gel and transferred to nitrocellulose. Each lane contained 10 μg of DNA, and each digest was done in duplicate. The probe used was a coding-sequence fragment from the *D. melanogaster* hsp83 clone, pPW244, from the Oregon R strain (see text), labeled by the random-primer method (17). For the two Southern blots shown (6A and B), the hybridizations were carried out in 30% formamide at 37°C for 24 h, which constitutes moderate stringency conditions. Identical results were obtained by using 40% formamide at 37°C. Molecular sizes (in kilobases) are indicated at the left.

cells there is a slight enrichment of this protein in the cytoplasm (49). In antheridiol-treated cells, however, there is a rapid accumulation of the newly synthesized 85-kDa protein in the nucleus, followed by a subsequent diminution in the nucleus, with a concomitant increase in the cytoplasm (10). It is unlikely that the presence of this protein in both the cytoplasmic and nuclear fractions reflects only cross-contamination of the fractions, since other prominent cytoplasmic proteins found in hormone-treated cells, such as the 24.3-kDa hormone-regulated protein (10), are not detected in the nuclear fraction, and prominent nuclear proteins, such as the 24- to 28-kDa *Achlya* heat shock proteins, are not detected in the cytoplasmic fraction (49). At present, there

does not appear to be a consensus with respect to the cellular localization of hsp90. For example, several studies have reported hsp90 to be an exclusively cytoplasmic protein (13, 27, 51, 56), while other studies reported that hsp90 was both a cytoplasmic and a nuclear protein (6, 18). In *A. ambisexualis*, both the steroid-induced and the heat shock-induced 85-kDa proteins are observed in both the nuclear (Fig. 1) and cytoplasmic (10, 49) fractions.

In vitro translation studies in which RNA from heat shocked and hormone-treated *Achlya* mycelia was used showed that with either treatment, there appeared to be an increase in the accumulation of mRNA encoding a similar 85-kDa protein. These 85-kDa translation products had similar but not identical electrophoretic behaviors relative to their respective in vivo 85-kDa proteins. On two-dimensional gels, the in vivo proteins focus to the acidic range of the gels, while the 85-kDa in vitro translation products exhibit a streaked appearance. The electrophoretic behavior of these translation products is not altered by treatment of the translated protein samples with RNase A or alkaline phosphatase. The basis of this electrophoretic behavior is not known at present; however, it appears to be a characteristic feature of the 85-kDa in vitro translation products.

Northern blot analyses confirmed the conclusions of the in vitro translation studies. An mRNA complementary to *Drosophila* hsp83, with an approximate size of 2.8 kilobases, shows increased accumulation in both heat-shocked and hormone-treated cells. This size is consistent with hsp90 mRNA from other organisms. This increased mRNA accumulation is also consistent with in vivo protein synthesis studies which suggested that both heat shock and hormone induce the increased synthesis of 85-kDa proteins. Taken together, these results suggest that the 2.8-kilobase *Achlya* mRNA which hybridizes to the *Drosophila* hsp83 probe encodes the 85-kDa heat shock and hormone-regulated protein. These results do not rule out the possibility that the observed increase in these mRNAs might be due to an increase in message stability. However, studies with dactinomycin suggest that *Achlya* hsp85 is regulated at least to some extent at the level of transcription (data not shown).

There are only limited reports (25, 36, 46, 52) of hsp90 family proteins inducible with steroid hormone, as is the case in *A. ambisexualis*. It is not known at this time whether the *Achlya* hormone- and heat shock-regulated 85-kDa proteins are encoded by the same or different genes. There are at least two different *Achlya* hsp85 sequences (12). These results are consistent with studies which have shown at least two hsp90 genes in HeLa cells (19), in the yeast *S. cerevisiae* (8), and in mouse cells (5). In *D. melanogaster*, however, the hsp83 protein appears to be encoded by only a single gene (20). Whether each of the *Achlya* hsp85 gene sequences is regulated by both stress and steroid hormone remains to be determined.

It is of interest that a number of steroid receptors, including the receptors for progesterone (15, 24; reviewed in reference 35), estrogen (24, 37), androgen (24), and glucocorticoid (42–44; reviewed in reference 35), have been shown to be complexed with an acidic protein in the molecular-weight range of approximately 90,000. This 90-kDa protein does not bind the steroid ligand and is therefore sometimes referred to as a non-hormone-binding component of the steroid receptor complex. An antigenically related 90-kDa phosphoprotein is found also in association with the transforming protein pp60^{src} of Rous sarcoma virus (45) and the tyrosine kinases of several other transforming viruses (29, 58). In these vertebrate systems, this 90-kDa protein has been identified as the heat shock protein, hsp90 (15, 23, 42, 43, 45, 57). The monoclonal antibody AC88, prepared against an *Achlya* receptor-associated protein, has been shown to cross-react with the 90-kDa non-hormone-binding component of a variety of vertebrate steroid receptors (38). An 85-kDa protein recognized by monoclonal antibody AC88 is observed in *Achlya* control mycelia and shows increased accumulation with heat shock (Fig. 4). An 85-kDa in vitro translation product of RNA from heat-shocked or hormone-treated cells could be immunoprecipitated with AC88. Thus, both the in vivo- and in vitro-translated heat shock and hormone-regulated 85-kDa proteins are antigenically related to one another and to the non-hormone-binding component of the putative *Achlya* steroid receptor. These results suggest that as is true in more complex mammalian and avian systems, the *Achlya* steroid receptor is associated with a member of the hsp90 family of proteins. In *A. ambisexualis*, this 85-kDa receptor component is itself inducible by steroid hormone.

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