A Subpopulation of the v-erb A Oncogene Protein, a Derivative of a Thyroid Hormone Receptor, Associates with Heat Shock Protein 90*

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The v-erb A oncogene of avian erythroblastosis virus is derived from a host gene for a thyroid hormone receptor and is able to block differentiation of erythroid cells and modify the growth properties of fibroblasts. Unlike its host cell progenitor, the v-erb A protein is found in both cytoplasmic and nuclear fractions of the cell. I report here that the cytoplasmic form of the v-erb A protein is associated in a higher molecular weight complex with heat shock protein 90, the same polypeptide found in association with the unliganded steroid receptors and with the soluble forms of the src oncogene.

The molecular basis by which the steroid and thyroid hormone receptors are regulated by hormone is poorly understood. Binding of cognate hormone at physiological temperatures results in a transformation of these receptors into active transcriptional factors, but the actual events associated with this "transformation" are poorly defined and appear to differ among the different receptors (reviewed in Sherman and Stevens, 1984; Yamamoto, 1985; Pratt, 1987; and Beato, 1989). For example, in the absence of cognate hormone, the glucocorticoid receptor appears to exist largely in a cytoplasmic form (Picard and Yamamoto, 1987), whereas the estrogen and progesterone receptors appear to be constitutively nuclear, possibly soluble, polypeptides (Clark, 1985; Guiochon-Mantel et al., 1989; Picard, et al., in press), and the thyroid hormone receptors are thought to be tightly bound to chromatin (reviewed in Apriletti et al., 1983). Binding of appropriate hormone results in activation of all of these receptors, a process associated with translocation of the (glucocorticoid) receptor into the nucleus, binding of the receptors to specific DNA sequences, and activation or repression of transcription of adjacent target genes (Sherman, 1984; Yamamoto, 1985; Picard and Yamamoto, 1987; Pratt, 1987; Beato, 1989). A common theme among all the steroid receptors examined is that in the absence of ligand, the receptor is found in a high molecular weight complex with a heat shock protein, HSP 90; binding of hormone results in correlation with dissociation of the receptor-HSP 90 complex (Grandics et al., 1984; Joab et al., 1984; Sanchez et al., 1985; Mendel et al., 1986; Denis et al., 1987, 1988; Toft et al., 1987; Aranyi et al., 1988; Revin et al., 1988). The exact role of HSP 90 in the receptor transformation process is unknown, but the commonality of this association among the different steroid receptors suggests an important function; perhaps HSP 90 maintains these receptors in an inactive form in the absence of hormone or aids in proper folding or assembly of the nascent receptor molecules (reviewed in Denis and Gustafsson, 1989). In contrast with these observations on the steroid receptors an association of the thyroid hormone receptors with HSP 90 has neither been detected nor, due to the apparently constitutive association of these receptors with chromatin, anticipated (Dalman et al., 1990).

The v-erb A oncogene of avian erythroblastosis virus (AEV) is a virally transduced, altered version of a host cell locus (c-erb A) encoding a thyroid hormone (T3/T4) receptor (Sap et al., 1986; Weinberger et al., 1986). The v-erb A oncogene blocks differentiation of immature erythroid cells and alters the growth properties of fibroblasts (Graf and Beug 1983; Gandrillon et al., 1987). Due to both deletion and point mutations relative to the host cell progenitor, the v-erb A protein neither binds thyroid hormone nor acts as a transcriptional activator in animal cells; instead, the v-erb A protein appears to interact with thyroid hormone response elements as a competitive repressor of T3/T4 receptor action (Munoz et al., 1988; Damm et al., 1989; Sap et al., 1989).

We are interested in elucidating differences in the properties of the viral and cellular erb A proteins so as to understand the molecular basis for the conversion of the T3/T4 receptor into an "oncogene." One of the significant differences between the v- and c-erb A polypeptides is that although all of the endogenous c-erb A protein and a subpopulation of the v-erb A protein appear to be constitutively nuclear polypeptides, a significant fraction of the viral erb A protein (30-40%) also exists as a stable cytoplasmic species of unknown function (Bigler and Eisenman, 1988; Boucher et al., 1988). We report here that the cytoplasmic v-erb A protein species can be found in a high molecular weight complex with HSF 90, an association which was unanticipated from previous models of action of this hormone-independent viral polypeptide. Possible implications for the mechanism of action of v-erb A in establishing the oncogenic phenotype are discussed.

**EXPERIMENTAL PROCEDURES**

**Cells, Virus, and Immune Reagents—** Chicken erythroblast cells transformed by the ES-4/R strain of avian erythroblastosis virus were maintained in Dulbecco's modified Eagle's medium supplemented with 10% tryptose phosphate broth (Difco), 8% fetal bovine serum 1% heat-inactivated chicken serum, 100 units of penicillin/ml, 1 mg of streptomycin/ml, and 2.5 μg of amphotericin B/ml in a 5% CO2 atmosphere. For cross-linking and immunoprecipitation experiments approximately 1×106 infected cells were radiolabeled with 500 μCi o Tran35S-label (ICN Radiochemical Co.) in 5 ml of RPMI medium lacking unlabeled methionine and cysteine (GIBCO). The cells were incubated for 3 h at 39°C and were subsequently washed twice in ice cold phosphate-buffered saline before lysis and immunoprecipitation analysis. For pulse-chase experiments, the cells were labeled with

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The abbreviations used are: AEV, avian erythroblastosis virus; DSP, dithiobis(succinimidyl propionate); EGS, ethylene glyco bis(succinimidyl succinate); SDS, sodium dodecyl sulfate.
Tran"S-label for 30 min and then washed twice with complete medium lacking radiolabel before the incubation was continued in complete medium for the indicated length of the chase period. For induction of heat shock proteins, the cells were transferred to 45 °C for 3 h prior to the addition of radiolabel and then incubated for 2 h more at 45 °C before harvest and lysis.

Antiserum directed against the v-erb A protein was prepared as previously described (Bonde and Privalsky, 1986). Antibodies to heat shock protein 90 were the generous gifts of Dr. David Toft (Mayo Medical School) and Dr. Joan Brugge (University of Pennsylvania School of Medicine).

Cell Lysis and Subcellular Fractionation—All manipulations were performed at 0 or 4 °C. Cell pellets representing 10" AEV-infected erythroid cells were resuspended in 400 µl of PTM buffer (20 mM potassium phosphate, pH 7.5, 0.5% Triton X-100, and 1 mM MgCl₂). For preparation of whole cell lysates, the resulting cell suspension was immediately lysed by two 15-s bursts in a Branson sonifier with cup horn at full power, and insoluble debris was removed by a 30-s centrifugation at 12,000 × g. For preparation of subcellular fractions, the cell pellets were resuspended in the PTM buffer and incubated for 15 min; the nuclei were subsequently pelleted at 12,000 × g for 10 s. The supernatant (representing the cytoplasmic and solubilized membrane fractions) was saved, and the nuclei were washed once and resuspended in 400 µl of PTM buffer. The final nuclear fraction was sonicated and clarified by centrifugation as described above for whole cells.

Cross-linking Protocols—We applied to the v-erb A protein a protocol developed for chemical cross-linking of the progesterone receptor (Rexin et al., 1988). Dithiobis(succinimidyl propionate) (DSP) or ethylene glycolbis(succinimidy1 succinate) (EGS) was freshly prepared as 100 mg/ml stocks in Me₂SO. The chemical cross-linker was added to the radiolabeled cell extracts to a final concentration of 1 µg/ml, and the mixture was incubated on ice for 30 min. A precipitate usually formed which was removed at the end of the incubation period by a 1-min centrifugation at 12,000 × g. Unreacted cross-linker in the cell extract was quenched by the addition of lysine to 30 mM, and the sample was incubated for an additional 20 min. Control samples were mock-treated with Me₂SO in the absence of cross-linker and were processed in parallel.

Immunoprecipitations, Column Analysis, and Peptide Mapping—Mock or cross-linked cell extracts (200 µl each) were mixed with an equal volume of TNTB buffer (10 mM Tris-Cl, pH 7.5, 100 mM NaCl, 1% Triton X-100, and 1 mg/ml bovine serum albumin) and 5 µl of antiserum/sample. After a 30-min incubation on ice, 25 µl of packed protein A-Sepharose (Sigma) in 1 ml of TNTB buffer were added to each sample, and the incubation was continued for 15 min on an inverting miter. The resulting immunocomplexes were collected by a 10-s centrifugation in a microcentrifuge; washed with four 1-ml changes of 10 mM Tris-Cl, pH 7.5, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 0.3% SDS; and washed once with 10 mM Tris-Cl, pH 7.5. Radiolabeled proteins in the immunocomplexes were resolved by SDS-polyacrylamide gel electrophoresis and were detected by fluorography (Privalsky et al., 1983).

Fractionation of cross-linked proteins by Sephacryl 400 chromatography was performed in a 12-ml bed volume column preequilibrated with 10 mM Tris-Cl, pH 7.5, 200 mM NaCl, 0.5% Triton X-100, and 1 mg/ml bovine serum albumin. Mock or cross-linked extracts were adjusted to 200 mM NaCl and were applied to the column in a 200-µl volume; 0.5-ml fractions were collected and were analyzed by the immunoprecipitation procedure described above. The column was calibrated by use of protein molecular weight standards as indicated in Fig. 2.

Peptide mapping by partial proteolysis was performed as previously described, using V8 and papain proteases (Privalsky et al., 1983).

RESULTS

The v-erb A Protein Is Associated with HSP 90

The v-erb A Protein in AEV-Infected Cells Can Be Isolated as a High Molecular Weight Complex by Chemical Cross-linking—We wished to investigate possible associations of the AEV v-erb A protein with either host polypeptides or itself, into higher order complexes. To stabilize these putative multicomponent complexes, we used a chemical cross-linking procedure. AEV-infected erythroid cells were radiolabeled with "S-aminoacids and lysed in low ionic strength buffer, and the lysates were treated with DSP or EGS (both are homobifunctional N-hydroxysuccinimide ester cross-linkers differing principally in the length and nature of their linker arms) (Lomant and Fairbanks, 1976; Abdella et al., 1979). The lysates were next immunoprecipitated with v-erb A-directed sera, and the polypeptides in the immunoprecipitants were analyzed by nonreducing SDS-polyacrylamide gel electrophoresis. Fig. 1A displays the results of such a procedure and demonstrates that the M, 75,000 v-erb A protein was virtually quantitatively cross-linked into an extremely large complex by either DSP or EGS treatment (compare lanes 4, 5, and 6). This complex barely entered the 8% polyacrylamide gel used in this analysis, indicating a relative molecular mass of over 200,000 Da. Control analyses of mock-treated cell extracts or of cross-linked extracts immunoprecipitated with preimmune sera exhibited little or no evidence of this high molecular mass material (Fig. 1A, lanes 1–3).

A M, 90,000 Protein Is Associated with the v-erb A Complex—To investigate the components of the large protein complex identified by DSP treatment, the cross-linked material was immunoprecipitated with v-erb A-directed antiserum and was reduced with 4% mercaptoethanol before SDS-polyacrylamide gel electrophoresis (Fig. 1B). The M, 75,000 v-erb A protein monomer was quantitatively recovered by this procedure, confirming the reversibility of the cross-linking. A number of smaller proteins were also found in these immunoprecipitates in the presence or absence of cross-linker (indicated by arrowheads); these smaller polypeptides have been previously demonstrated to be closely related to the full-length v-erb A protein in AEV-infected cells (Lomant and Fairbanks, 1976; details described in Fig. 2).

FIG. 1. Immunoprecipitation analysis of chemically cross-linked complexes of the v-erb A protein. Panel A, SDS-polyacrylamide gel electrophoresis under nonreducing conditions. AEV-infected cells were metabolically radiolabeled with "S-aminoacids and lysed by sonication, and the cell extracts were treated with Me₂SO alone (lanes 1 and 4), 1 mg/ml DPS (lanes 2 and 5), or 1 mg/ml EGS (lanes 3 and 6) as described under “Experimental Procedures.” The cross-linked polypeptides were subsequently immunoprecipitated with either preimmune (lanes 1–3) or erb A-directed (lanes 4–6) sera, and the immunocomplexes were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. The positions of proteins run as size markers in an adjacent lane (20S, 116, 97.4, 65, 45, and 29 Da from top to bottom) are indicated. Panel B, SDS-polyacrylamide gel electrophoresis after reduction. Radiolabeled cell extracts were prepared as described for panel A and either treated with 1 mg/ml DSP (lanes 1 and 2) or mock-treated (lanes 3 and 4) prior to immunoprecipitation with preimmune (lanes 1 and 3) or erb A-directed (lanes 2 and 4) antiserum. Each immunoprecipitate was reduced with 4% mercaptoethanol prior to SDS-polyacrylamide gel electrophoresis and fluorography.
v-erb A protein and probably represent proteolysis products or internal initiations on the mRNA (Bigler and Eisenman, 1988; Boucher et al., 1988). In contrast to these bands, a 35S-labeled protein approximately 90,000 Da in relative molecular mass appeared to be immunoprecipitated only with v-erb A-directed sera from DSP-treated cells (Fig. 1B, lane 2, 90 kD) and was not found in identical immunoprecipitates in the absence of cross-linker (lane 4) or in immunocomplexes formed by preimmune serum (lanes 1 and 3). In addition, a second protein, approximately 59,000 Da in relative molecular mass, was often detected in the cross-linked and reduced erb A immunoprecipitates (Fig. 1B, lane 2, 59 kD). A polypeptide of similar size has been reported in steroid receptor complexes (Tai et al., 1986; Toft et al., 1987). However, our recovery of this latter protein was low, and a detailed characterization of the M, 59,000 polypeptide was not performed in this study.

Column Analysis of the Components of the v-erb A Complex—To characterize better the size and composition of the high molecular weight v-erb A protein complex, DSP cross-linked cell extracts were applied to a Sephacryl 400 column in high salt (200 mM NaCl) buffer, and the resulting column fractions were immunoprecipitated, reduced with 2-mercaptoethanol, and analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2B). For comparison, an identical column fractionation was performed on mock-treated cell extracts (Fig. 2A). Under these salt conditions, the majority of the mock-treated v-erb A protein fractionated in a position consistent with a monomer, eluting with a peak between that of the phosphorylase a (97.4-kDa monomer) and bovine serum albumin (65-kDa monomer) markers. In contrast, pretreatment of the infected cell lysate with DSP resulted in a significant displacement of the v-erb A protein elution profile toward higher molecular weights, resulting in some proportion of the v-erb A protein eluting in the void volume (approximately 400,000 Da in relative molecular mass). Once again, an approximately 90-kDa protein was detected in association with the cross-linked v-erb A protein, eluting from the column with a molecular mass consistent with a 90-kDa protein v-erb A protein heterodimer (fractions 14 and 15). No 90-kDa polypeptide was immunoprecipitated with the v-erb A protein in the absence of cross-linker (panel A) or with preimmune sera (data not shown).

Subcellular Fractionation—At steady state, approximately 60–70% of the v-erb A protein is present in the nucleus of infected cells, whereas 30–40% exists as a cytoplasmic species of unknown function (Boucher et al., 1988). We therefore separated AEV-infected cells into nuclear and cytoplasmic (plus membrane) fractions prior to treatment with cross-linker and subsequent immunoprecipitation analysis (Fig. 3). Both cytoplasmic and nuclear forms of the v-erb A protein could be cross-linked as a high molecular weight complex by DSP (data not shown), but only the cytoplasmic form yielded a 90-kDa host protein upon immunoprecipitation and reduction (Fig. 3, lane 4). Once again, neither preimmune sera (Fig. 3, odd-numbered lanes) nor v-erb A-directed sera in the absence of cross-linker (Fig. 3, lanes 2 and 6) precipitated the 90-kDa protein, confirming the specificity of the association.

A two-dimensional analysis of the cytoplasmic v-erb A protein complex is presented in Fig. 4. The first dimension was a nonreducing SDS-polyacrylamide gel lane which was subsequently excised and reduced, and the proteins were resolved by electrophoresis on a second SDS-polyacrylamide gel. The v-erb A protein not subjected to the cross-linking procedure (panel A, open arrow) and the v-erb A-related small polypeptide fragments discussed previously ran as spots

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**Fig. 2.** Sephacryl 400 column fractionation of cross-linked and non-cross-linked v-erb A protein complexes. Extracts of 35S-aminocids radiolabeled, AEV-infected cells were either mock-treated (panel A) or cross-linked with 1 mg/ml DSP (panel B), and the extracts were applied to a Sephacryl 400 column as described under "Experimental Procedures." Fractions collected from the column were immunoprecipitated with antisera directed against the v-erb A protein, and the immunoprecipitated polypeptides were analyzed by SDS-polyacrylamide gel electrophoresis after reduction with 2-mercaptoethanol. The column was calibrated by fractionation of blue dextran, β-galactosidase (tetramer of 464 kDa), myosin (205 kDa), phosphorylase a (97.4 kDa), bovine serum albumin (65 kDa), and carbonic anhydrase (29 kDa) marker proteins, as indicated.

**Fig. 3.** Analysis of v-erb A protein complexes in isolated nuclear and cytoplasmic fractions. 35S-Aminoacid radiolabeled, AEV-infected cells were fractionated into nuclear and cytoplasmic/membrane fractions by detergent lysis. Each fraction was either mock-treated (lanes 1, 2, 5, and 6) or treated with 1 mg/ml DSP (lanes 3, 4, 7, and 8), and the proteins were immunoprecipitated using preimmune (odd-numbered lanes) or v-erb A-directed (even-numbered lanes) serum. Proteins in the immunoprecipitates were reduced, resolved by SDS-polyacrylamide gel electrophoresis, and visualized by fluorography.
A. NO CROSSLINKER

B. DSP TREATED

Fig. 4. Two-dimensional gel electrophoresis analysis of cytoplasmic v-erb A protein complexes. Cytoplasmic extracts were prepared from 35S-aminoacids radiolabeled, AEV-infected cells and either mock-treated (panel A) or treated with 1 mg/ml DSP (panel B). The extracts were subsequently immunoprecipitated with sera directed against the erb A protein, and the immunoprecipitates were resolved by electrophoresis under nonreducing conditions on a SDS-7% polyacrylamide gel. This first dimension was subsequently equilibrated for 20 min in 4% 2-mercaptoethanol, 66 mM Tris-CI, pH 6.8, 1% sodium dodecyl sulfate, and 10% glycerol and then sealed across the top of a SDS-6% polyacrylamide gel. After electrophoresis in the second dimension, the gels were stained, fixed, and fluorographed. The positions of unlabeled proteins included as internal size standards are indicated (1, β-galactosidase (116 kDa); 2, phosphorylase a (97.4 kDa); 3, bovine serum albumin (65 kDa); 4, ovalbumin (45 kDa); 5, carbonic anhydrase (29 kDa)). The position of the 75-kDa v-erb A protein monomer is indicated by an open arrow, the positions of dimeric and higher multimeric forms by closed arrows, and the position of the 90-kDa host protein by a diamond.

aligned on a diagonal, as expected for proteins not affected by reduction. In contrast, the majority of the DSP-cross-linked v-erb A protein ran as high molecular weight complexes in the first dimension which, after reduction, could be resolved into the 75-kDa monomeric form of the v-erb A protein and the 90-kDa host polypeptide (panel B, diamond and closed arrows). Monomeric forms of the v-erb A protein (panel B, open arrow), as well as possible dimeric forms, could also be detected in the electrophoretogram. This analysis demonstrated that a significant portion of the v-erb A protein complex in the cytoplasmic fraction consisted of the v-erb A protein itself and the 90-kDa host polypeptide, but due to differences in pool sizes and in the efficacy of cross-linking, the exact stoichiometry of these two components could not be readily determined. A similar analysis of the cross-linked nuclear v-erb A protein complex yielded only the monomeric v-erb A protein on reduction (data not shown), suggesting that the nuclear complex may represent a homomeric association of this polypeptide.

The 90-kDa Polypeptide Associated with the v-erb A Protein Is Heat Shock Protein 90—Due to the known association of HSP 90 with steroid receptors (reviewed in Toft et al., 1987), we tested to determine if the 90-kDa polypeptide cross-linked to the v-erb A protein was, in fact, chicken HSP 90. Avian erythroid cells were incubated at 45 °C to induce the heat shock response, the cells were metabolically radiolabeled with 35S-aminoacids, and the proteins synthesized were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 5A). Heat shock resulted in enhanced synthesis of four proteins, 90,000/89,000, 73,000, 35,000, and 27,000 Da in relative molecular mass (compare lanes 1 and 2), as previously reported (Oppermann et al., 1981; Lindquist and Craig, 1988). Synthesis of the same proteins was also stimulated by arsenite treatment (data not shown), another known inducer of the “heat shock” response (Lindquist and Craig, 1988). The 90-kDa polypeptide induced by heat shock (lane 2) migrated with the same apparent molecular mass as the 90-kDa polypeptide associated with the v-erb A protein (lane 4); this is most clearly seen in lane 5, for which aliquots of the two different samples were mixed prior to electrophoresis.

To confirm the identity of the 90-kDa heat-induced and the 90-kDa v-erb A-associated polypeptides, the proteins were excised from the gel and subjected to partial proteolysis peptide mapping (Fig. 5A, inset). Using two different concentra-

FIG. 5. Comparison of the v-erb A protein-associated polypeptide with heat shock protein 90. Panel A, comparison with heat shock-induced proteins. Cells were radiolabeled with 35S-aminoacids either at 39 °C (lane 1) or 45 °C (lane 2), and equal trichloroacetic acid-precipitable counts were analyzed by SDS-polyacrylamide gel electrophoresis. The positions of the major heat-inducible proteins are indicated, in kDa, to the left of the figure. In a parallel experiment, radiolabeled cytoplasmic extracts of infected cells were cross-linked with DSP and immunoprecipitated with preimmune (lane 3) or anti-erb A antisera (lane 4) and were analyzed on the same gel. A mixture of the samples from lanes 2 and 4 was also analyzed (lane 5). For comparison, infected cell extracts mock-treated with MeSO alone and immunoprecipitated with preimmune (lane 6) or anti-erb A (lane 7) sera were characterized in parallel lanes. Inset, the 90-kDa proteins isolated either as heat-induced polypeptides from whole cells (lanes a and d) or as polypeptides cross-linked with the v-erb A protein complex (lanes b and e) were excised from the gel and analyzed by partial proteolysis peptide mapping. Protease fragments of the p75v-erb A protein (lanes c and f) were compared in parallel. Either 0.2 μg (lanes a–c) or 2 μg (lanes d–f) of V8 protease were used per lane of the peptide map. Panel B, comparison with immunoprecipitated HSP 90. 35S-Aminoacid radiolabeled cell lysates were immunoprecipitated with normal rabbit serum (lane 1) or with two different HSP 90-directed monoclonal antibodies, AC88 (lane 3) and D7a (lane 4). In parallel, radiolabeled cytoplasmic extracts of infected cells were cross-linked with DSP and immunoprecipitated with anti-erb A (lane 2) antisera. Inset, the 90-kDa proteins in lanes 2–4 were subsequently characterized by partial V8 protease mapping (lanes a–c, respectively) using 2 μg of protease/sample.
tions of V8 protease, the two 90-kDa proteins yielded peptide maps that were identical to one another, yet distinct from those generated by the v-erb A protein itself (compare lanes a-c and d-f). Identity of the 90-kDa v-erb A-associated and the 90-kDa heat-induced proteins was also found by using papain in the partial proteolysis mapping technique (data not shown).

We also compared the 90-kDa v-erb A-associated protein to authentic HSP 90 isolated by immunoprecipitation with specific antisera (Fig. 5B). Two different HSP 90-directed monoclonal antibodies were employed; both precipitated an identical 90-kDa protein (lanes 3 and 4) that migrated at a position identical to that of the v-erb A-associated protein (lane 2) and which was not recognized by normal rabbit serum (lane 1). Once again, partial proteolysis maps (Fig. 5B, inset, and data not shown) confirmed the identity of the polypeptides recognized by the HSP 90-directed antibodies (lanes b and c) with the v-erb A-associated 90-kDa polypeptide (lane a).

To confirm independently the association of v-erb A protein with HSP 90 and to compare the components of the v-erb A protein complex with others of known HSP 90 polypeptide complexes, the ability of HSP 90-directed antibodies to function reciprocally in the coprecipitation of the v-erb A protein was assayed (Fig. 6). In the absence of chemical cross-linker, two monoclonal antibodies directed against the HSP 90 protein, D7a and AC88, recognized the HSP 90 protein with high specificity (Fig. 6, lanes 4 and 5). If the cell lysates were pretreated with DSP cross-linker, the D7a antibodies also coprecipitated the p75\textsuperscript{erb A} protein, along with additional polypeptides approximately 73, 68, and 59 kDa in apparent molecular mass (lane 5). Detectable, although much lower, levels of these additional polypeptides were also immunoprecipitated by the HSP 90-directed D7a serum in the absence of DSP cross-linker (lane 4, and results not shown), indicating that coprecipitation of these proteins is not simply an artifact of chemical cross-linking but reflects the presence of a preexisiting complex which is stabilized by the DSP cross-linker. In contrast to D7a, the AC88 monoclonal antibody is unable to recognize HSP 90 molecules complexed to other proteins (Kost et al., 1989); consistent with this observation, none of the HSP 90-associated proteins were coprecipitated by AC88 antibodies in either the presence or absence of DSP cross-linker (lanes 6 and 7).

The 59-kDa polypeptide coprecipitated by use of HSP 90 antibodies appeared to be the same protein as that coprecipitated by erb A-directed antibodies (Fig. 1B, lane 2, and Fig. 6, lane 3), whereas the 73- and 68-kDa polypeptides were not found in immunoprecipitates mediated by erb A-directed serum (Fig. 6, lane 3). The 73- and 68-kDa polypeptides, therefore, presumably form discrete complexes with the HSP 90 protein and are not components of the v-erb A protein-HSP 90-59-kDa polypeptide complex. The approximately M, 73,000 protein may be HSP 70, a polypeptide previously reported as a component of certain HSP 90 protein-steroid receptor complexes (Wrangle et al., 1988; Kost et al., 1989). The identity of the 68-kDa protein is unknown.

**Pulse-Chase Experiments Suggest a Stable Association between v-erb A Protein and HSP 90**—The stability of the HSP 90-v-erb A complex was examined by pulse-chase analysis (Fig. 7). After a 30-min pulse label with \textsuperscript{35}S-aminoacids, AEV-infected cells were chased for various lengths of time with unlabeled medium and lysed, and the lysates were treated with DSP and immunoprecipitated with erb A-directed or HSP 90-directed antibodies (Fig. 7). Measuring complex formation either as the amount of HSP 90 coprecipitated by erb A-directed serum (Fig. 7, lanes 1-6) or as the amount of v-erb A protein coprecipitated by HSP 90-directed antibodies (lanes 7-12) yielded similar conclusions; the v-erb A protein appeared to enter into the HSP 90 complex within the first 30 min, either cotemporaly or very rapidly after v-erb A protein synthesis. Once in complex with the HSP 90 protein, the v-erb A protein demonstrated a half-life of 110 min, a turnover rate virtually identical to that of the bulk v-erb A population (half-life of 120 min), suggesting that the stability

![Fig. 6. Coprecipitation of the v-erb A protein by use of HSP 90-directed antibodies. AEV-infected erythroid cells were radio-labeled with \textsuperscript{35}S-aminoacids and lysed, and the lysates were either treated with DSP cross-linker (lanes 1, 3, 5, and 7) or mock-treated (lanes 2, 4, and 6). The lysates were subsequently immunoprecipitated with erb A-directed antibodies (lanes 2 and 3), with preimmune antibodies from the same rabbit (lane 1), or with HSP 90-directed antibodies, either a monoclonal antibody that recognizes HSP 90 in complex with other proteins (D7a, lanes 4 and 5) or a monoclonal antibody that only recognizes free HSP 90 molecules (AC88, lanes 6 and 7). The immunoprecipitates were resolved by SDS-polyacrylamide electrophoresis and visualized by fluorography. The positions of molecular weight markers (116, 84 and 58 kDa), analyzed in parallel, are indicated to the left of the figure, and the positions of the HSP 90, p75\textsuperscript{erb A}, and the 59-kDa protein are indicated to the right.](image)

![Fig. 7. Pulse-chase analysis of v-erb A protein-HSP 90 complex formation. AEV-infected erythroid cells were pulse-labeled for 30 min with \textsuperscript{35}S-aminoacids and then either immediately harvested (lanes 1 and 7) or washed and incubated with unlabeled medium for an additional 30 (lanes 2 and 8), 90 (lanes 3 and 9), 150 (lanes 4 and 10), 300 (lanes 5 and 11), or 480 (lanes 6 and 12) min at 39°C. The cells were collected and lysed, and the cytoplasmic fractions were cross-linked with DSP. The cross-linked lysates were subsequently immunoprecipitated with either erb A-directed antisera (lanes 1-6) or with HSP 90-directed D7a monoclonal antibodies (lanes 7-12), and the immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Positions of the p75\textsuperscript{erb A} and HSP 90 proteins are indicated.](image)
of the v-erb A protein is not noticeably altered by HSP 90 association. If, as has been suggested of steroid receptors, only de novo synthesized v-erb A protein associates with HSP 90, these results also indicate that the v-erb A protein-HSP 90 complex is relatively stable and lasts for the life of the v-erb A protein. The half-life of HSP 90 complexes containing the 73-, 68-, and 59-kDa proteins was even more stable than the HSP 90 complexes containing the v-erb A and 59-kDa proteins (Fig. 7, compare lanes 1–6 with lanes 7–12), again indicating that the former represent a population distinct from the latter.

DISCUSSION

We report here that the v-erb A protein of AEV can be cross-linked into large complexes ranging in size from 150,000 to 400,000 Da in relative molecular mass. Virtually all of the v-erb A protein can be cross-linked in this manner, suggesting that these complexes represent the major physiological form of the v-erb A protein in infected cell lysates. The predominant radiolabeled polypeptide component of these complexes, whether isolated from nuclei or cytoplasmic extracts, appears to be the v-erb A polypeptide itself; evidence for homodimer and higher order associations could be detected. In addition, the cytoplasmic population of the v-erb A protein is in association with a host polypeptide, HSP 90.

The complexes of v-erb A protein and HSP 90 that we detect are unlikely to be an artifact of the chemical cross-linking; some coprecipitation of v-erb A protein and HSP 90 was detected in the absence of chemical cross-linker (Fig. 6), and this noncovalent association appeared to be further stabilized by molybdate, a known stabilizer of the steroid receptor-HSP 90 complexes (data not shown). Are these complexes of physiological significance or do they form only upon cell lysis? Strongly suggestive of a physiological role is the fact that only the cytoplasmic form of v-erb A protein is found in a complex with HSP 90, whereas the nuclear form, isolated under the same buffer conditions, is not. Although the exact subcellular distribution of HSP 90 in unstressed cells is controversial, HSP 90 has been reported as present in both nuclear and cytoplasmic fractions (Gasc et al., 1990).

These cytoplasmic complexes of the v-erb A protein closely parallel those of the "untransformed" steroid receptors, which exist in the absence of hormone ligand as heteromeric complexes probably consisting of the receptor linked to two HSP 90 molecules (Denis et al., 1987; Aranyi et al., 1988; Rexin et al., 1988; Randanyi et al., 1989). Hormonal activation of the steroid receptors as functional transcriptional factors is responsive to the c- and v-erb A proteins, it has not yet been possible to test this hypothesis. Unlike the glucocorticoid receptor, the cytoplasmic v-erb A protein appears capable of binding to the appropriate DNA response elements (Bonde and Privalsky, 1990), but we cannot confirm that HSP 90 remains associated with the v-erb A protein during the course of this assay.

It is intriguing that other oncogene products, such as the src protein, appear to be associated with heat shock protein 90 (Oppermann et al., 1981; Brugge, 1986). In the case of src, the association with HSP 90 appears to represent a transient step in its biosynthesis and association with plasma membrane.

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Note added in proof—We have recently determined that v-erb A acts in yeast (S. cerevisiae) not as a repressor but as a transcriptional activator (Privalsky et al., in press). We are exploring the possibility that the different v-erb A responses in mammalian and yeast cells may be due to the different heat shock proteins present in these phylogenetically distinct backgrounds.

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