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A Subpopulation of the Avian Erythroblastosis Virus v-*erbA* Protein, a Member of the Nuclear Hormone Receptor Family, Is Glycosylated

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The v-*erbA* oncogene of avian erythroblastosis virus is derived from a cellular gene for a thyroid hormone (T4/T3 thyronine) receptor and encodes a DNA-binding protein found principally in the nucleus of the infected cell. I report here that a subpopulation of the v-*erbA* protein is glycosylated. The v-*erbA* protein, therefore, is another member of the newly recognized family of eucaryotic transcription factors and related polypeptides which are glycoproteins.

The v-*erbA* oncogene within the avian erythroblastosis virus R (AEV) genome appears to function in oncogenesis by blocking the differentiation of infected erythroid cells and by modifying the growth properties of fibroblasts (4, 6, 8). The v-*erbA* gene is derived from a host cell gene (c-*erbA*) for a thyroid hormone (T4/T3 thyronine) receptor, one of a family of hormonally regulated transcription factors which includes steroid and retinoid receptors (11, 14). The v-*erbA* protein is a modified derivative of the T4/T3 thyronine receptor, bearing an N-terminal domain derived from retroviral *gag* sequences and containing a number of point mutations and small deletions relative to the c-*erbA* gene product (11, 14). In common with the c-*erbA* progenitor, the v-*erbA* protein retains the ability to enter the nucleus of infected cells and to bind to DNA in vitro; the v-*erbA* protein, however, has lost the ability to bind T4/T3 hormone and may function in oncogenesis by interfering with the action of the normal host T4/T3 thyronine receptors (2, 3, 11, 15).

It has recently been noted that a number of eucaryotic transcription factors are glycosylated through O linkages on serines or threonines (7). The glycosylation of nuclear polypeptides is unanticipated, and the scope and function of this modification remain unclear. I report here that, although structurally unrelated to these previously characterized transcription factors, the AEV v-*erbA* protein also contains covalently bound carbohydrate. This observation extends the scope of this newly recognized nuclear protein modification to a member of the steroid-thyronine-retinoid receptor gene family.

Detection of glycosylated v-*erbA* protein species. To determine whether the v-*erbA* protein was glycosylated, I tested the ability of the polypeptide to bind to a number of lectins immobilized on Sepharose. Approximately 10^6 AEV-infected cells were metabolically radiolabeled with 50 μCi of ^{35}S-amino acids for 3 h (>1,000 Ci/mmol; Tran ^{35}S-label; ICN Biochemical) and then sonicated in 600 μl of binding buffer (10 mM Tris hydrochloride [pH 7.5], 200 mM NaCl, 1 mg of bovine serum albumin per ml, and 1% [wt/vol] Triton X-100 in the case of wheat germ agglutinin [WGA] lectin), and the clarified cell lysates were centrifuged for 1 min at 12,000 × *g* and incubated for 1 h at 4°C with the lectin-Sepharose. The lectin-Sepharose was washed with five changes of binding buffer, and any proteins remaining bound to the lectin were eluted with the appropriate sugar in 600 μl of binding buffer. Both bound and unbound fractions were immunoprecipitated with various antisera, and the immunoprecipitated proteins were resolved by sodium dodecyl sulfate (SDS)–8% polyacrylamide gel electrophoresis and detected by fluorography (2).

A reproducible subpopulation of the v-*erbA* protein bound to WGA-Sepharose under these conditions and was eluted by *N*-acetylglucosamine (Fig. 1, lanes 2 and 4; note that lanes 1 to 4 were fluorographed for a longer period than lanes 5 to 8). The 75,000-molecular-weight polypeptide bound by the lectin was the product of the v-*erbA* gene, as demonstrated by partial proteolysis mapping and by its recognition by both anti-virus *gag* and anti-*erbA* sera (Fig. 1) but not by preimmune sera (Fig. 2B). The $p75^{gag\text{–}v\text{-}erbA}$ protein did not bind to nonderivatized Sepharose (Fig. 1, lanes 1 and 3).

Infectious stocks of the replication-defective AEV genome contain a Rous-associated virus as a helper (5); my anti-virus *gag* antibody also recognizes the $Pr76^{gag}$ precursor protein doublet synthesized by the Rous-associated virus type 1 helper virus (Fig. 1, lanes 7 and 8). Virtually none of the $Pr76^{gag}$ protein doublet bound to the WGA-Sepharose (compare lanes 4 and 8), verifying that nonglycosylated polypeptides were not retained by the lectin. This observation also suggests, although it does not confirm, that the glycosyl moieties present in the $p75^{gag\text{–}v\text{-}erbA}$ protein are likely to be derived from *erbA*-, not *gag*-, encoded portions of the *gag*–v-*erbA* fusion polypeptide.

I next tested the interaction of the v-*erbA* protein with the WGA-Sepharose to learn whether this interaction exhibited the expected sugar specificity. Metabolically radiolabeled v-*erbA* protein was bound to a WGA-Sepharose column, and the column was sequentially eluted with various monosaccharides (Fig. 2A). The bulk of the v-*erbA* protein eluted only with *N*-acetylglucosamine (lanes 5 and 9), as anticipated for the reported specificity of this lectin (9).

To confirm that the glycosyl moiety was linked directly to the v-*erbA* protein rather than to a second polypeptide which indirectly mediated the binding of the v-*erbA* protein to the WGA-Sepharose, I used a coupled immunoprecipitation and blotting protocol. Immunoprecipitates of AEV-infected cell lysates were subjected to denaturing SDS-polyacrylamide gel electrophoresis and were blotted to a nitrocellulose membrane. The positions of glycosylated proteins were visualized by incubation with biotinylated WGA and peroxidase-conjugated avidin (7). A glycosylated polypeptide reacting with the biotinylated WGA and migrating at the

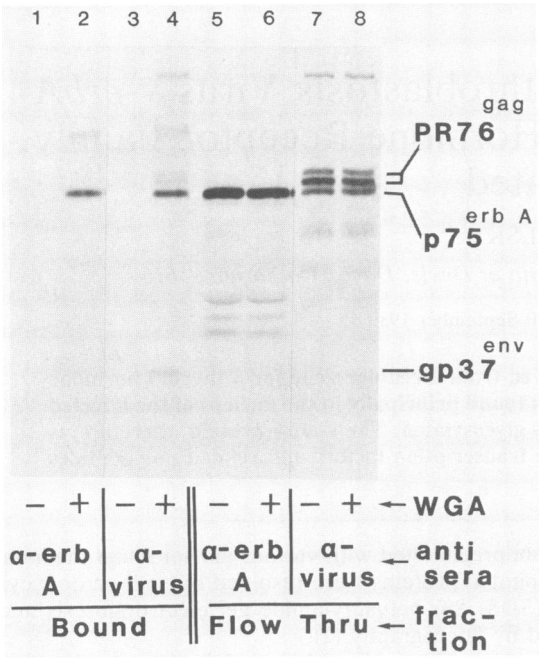

FIG. 1. Binding of the v-*erbA* protein to WGA-Sepharose. Extracts of ^{35}S-amino acid-labeled AEV-infected erythroid cells were incubated with 0.2 ml of Sepharose CL-4B (odd-numbered lanes) or 0.2 ml of WGA-Sepharose CL-4B (Sigma Chemical Co.) (5 mg of *Triticum vulgaris* lectin per ml of matrix; even-numbered lanes) as described in the text. Proteins not binding to the gel matrix were collected (lanes 5 to 8). After extensive washing, proteins remaining bound to the column were eluted with binding buffer containing 10% *N*-acetylglucosamine (lanes 1 to 4). All fractions were immunoprecipitated as previously described (2) with anti-*erbA* (α-erb A; lanes 1, 2, 5, and 6) or anti-virus (principally anti-*gag*-directed) (α-virus; lanes 3, 4, 7, and 8) antisera and were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography (2). Lanes 1 to 4 were exposed 10 times longer than lanes 5 to 8.

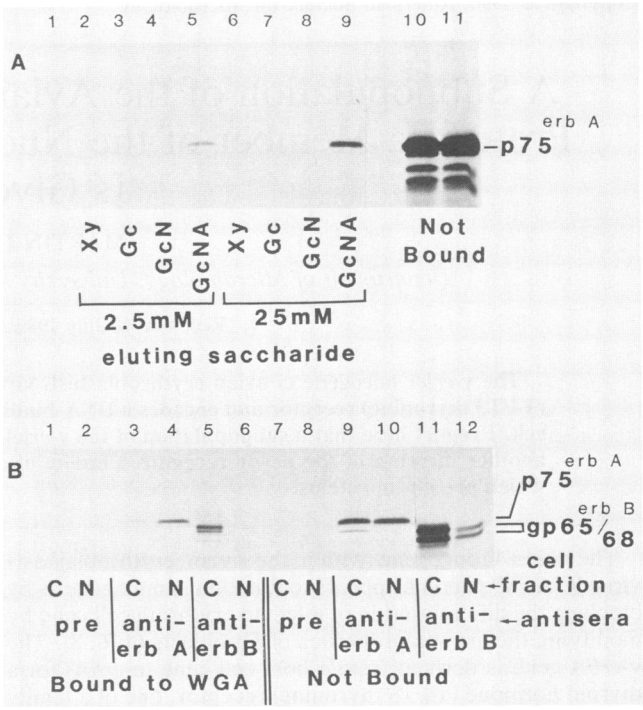

FIG. 2. (A) Specificity of elution of v-*erbA* protein with various monosaccharides. ^{35}S-amino acid-labeled cell lysates were incubated with WGA-Sepharose as described in the legend to Fig. 1. Proteins bound to the immobilized lectin were subsequently eluted with either 2.5 mM (lanes 2 to 5) or 25 mM (lanes 6 to 9) concentrations of the indicated monosaccharide: D-xylose (Xy), D-glucose (Gc), D-glucosamine (GcN), or *N*-acetyl-D-glucosamine (GcNA). The proteins eluted by these procedures were immunoprecipitated with anti-*erbA* serum and were analyzed by SDS-polyacrylamide gel electrophoresis. v-*erbA* protein not binding to the WGA-Sepharose (lane 11) and v-*erbA* protein binding (lane 1) or not binding (lane 10) to nonderivatized Sepharose are also shown. Lanes 1 to 9 were exposed five times longer than lanes 10 and 11. (B) Subcellular fractionation of glycosylated v-*erbA* proteins. AEV-infected erythroid cells were fractionated into nuclear (N) and cytoplasm-membrane (C) fractions by detergent lysis (2). The proteins in each fraction were incubated with WGA-Sepharose as described in the legend to Fig. 1, and the proteins in the bound (lanes 1 to 6) and unbound (lanes 7 to 12) fractions were immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis. Preimmune sera (pre; lanes 1, 2, 7, and 8), anti-*erbA* sera (lanes 3, 4, 9, and 10), and anti-*erbB* sera (lanes 5, 6, 11, and 12) were used in the immunoprecipitation analysis (2, 10).

position of the v-*erbA* protein was detected in immunoprecipitates of AEV-infected cells (Fig. 3, lanes 2, 4, and 5) but not in uninfected cells (lane 1). The identity of this glycoprotein as p75$^{gag-v\text{-}erbA}$ was confirmed by its immunoprecipitation by both *erbA*- and viral *gag*-directed sera (compare lanes 2, 4, and 5 with lane 6) but not by preimmune serum (lane 3). The rabbit immunoglobulins (also glycoproteins) used in the immunoprecipitations served as internal controls, reacting strongly with the biotinylated WGA used in lanes 1 to 5 as well as with the goat anti-rabbit immunoglobulin used in lane 6.

Time course and subcellular localization of the glycosylation. AEV-infected erythroid cells were pulse-labeled for 1 h with ^{35}S-amino acids at 39°C. The cells were then either immediately lysed (0 h) or washed and incubated in nonradioactive medium for 1-, 2-, 4-, 8-, or 24-h chase periods before lysis (2). The cell lysates were then incubated with WGA-Sepharose, and the proteins bound or not bound by the WGA-Sepharose were analyzed by immunoprecipitation with anti-*erbA* serum and by SDS-polyacrylamide gel electrophoresis (Fig. 4). Glycosylation of the v-*erbA* protein appeared to take place rapidly after its synthesis, within 1 to 2 h (Fig. 4A). The glycosylated form of the v-*erbA* protein also appeared to be slightly more stable than the bulk population in these experiments (Fig. 4; compare the 24-h chases in panels A and B).

The majority of the v-*erbA* protein is present in the nuclei of infected cells but that 30 to 40% of this protein is also stably present in the cytoplasm (1, 2). Subcellular fractionation (Fig. 2B) demonstrated that v-*erbA* proteins derived from both the nuclear and the cytoplasm-membrane (detergent-soluble) fractions of infected erythroid cells could bind to the WGA-Sepharose but that under steady-state conditions, proportionately more of the protein in the nuclear fraction bound to the lectin (compare lanes 3 and 4 with lanes 9 and 10). In contrast, the bulk of the glycosylated v-*erbB* protein, a transmembrane tyrosine kinase encoded by the second oncogene of AEV, was found in the cytoplasm-membrane fraction (lanes 5, 6, 11, and 12), as previously noted for this plasma membrane-associated glycoprotein (10).

Characterization of the glycosyl linkage. The previously characterized transcriptional factors are glycosylated through O linkages (7). O-linked carbohydrate is insensitive

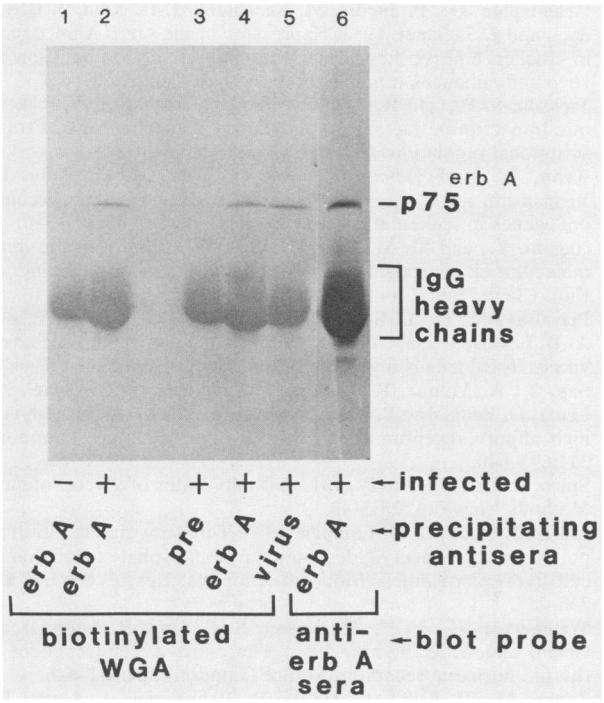

FIG. 3. Denaturing gel electrophoresis and blot analysis of glycosylated v-*erbA* proteins. Nonradioactive lysates of uninfected fibroblasts (lane 1), AEV-infected fibroblasts (lane 2), or AEV-infected erythroid cells (lanes 3 to 6), each representing approximately 750 μg of protein, were immunoprecipitated with preimmune (pre; lane 3), anti-*erbA* (lanes 1, 2, 4, and 6), or anti-virus *gag* (lane 5) antiserum. Proteins in the immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis and blotted to a nitrocellulose membrane, and the membrane was incubated with either biotinylated WGA and avidin-peroxidase to visualize glycosylated protein (7) (lanes 1 to 5) or with additional anti-*erbA* serum and anti-rabbit immunoglobulin G (IgG)-peroxidase (lane 6) to visualize the $p75^{gag-v\text{-}erbA}$ protein.

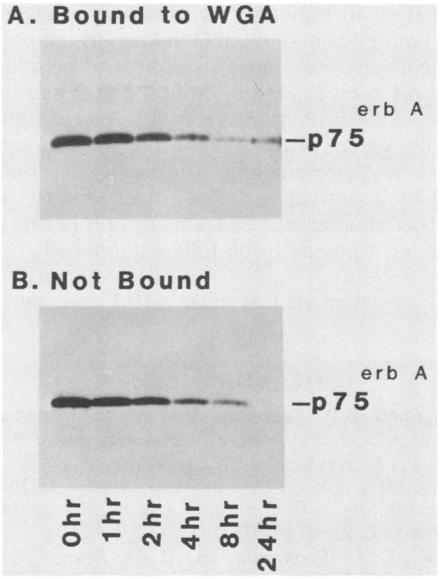

FIG. 4. Pulse-chase analysis of v-*erbA* protein glycosylation. AEV-infected erythroid cells were pulse-labeled for 1 h with ^{35}S-amino acids as described in the text and then chased with nonradioactive media for the indicated periods. The cells were then lysed, the lysates were incubated with WGA-Sepharose, and the radiolabeled v-*erbA* proteins bound (A) or not bound (B) by the lectin were analyzed as described in the legend to Fig. 1.

to treatment with tunicamycin, a specific inhibitor of N-linked protein glycosylation, but is sensitive to alkali hydrolysis (12, 13). AEV-infected erythroid cells were either treated or not treated with 1 μg of tunicamycin per ml for 4 h and were then radiolabeled with ^{35}S-amino acids in the same medium for an additional 2 h (10). The cells were then lysed, and the ability of the v-*erbA* protein to bind to WGA-Sepharose was determined (Fig. 5A). Consistent with O linkage, the glycosylation of the v-*erbA* protein was completely resistant to treatment with tunicamycin (Fig. 5A, lanes 1 and 2). Under the same conditions, glycosylation of the Rous-associated virus type 1 gp85 envelope protein in the same cell lysates (also detected by the anti-virus serum; Fig. 5A, lanes 3 and 4) was dramatically inhibited, confirming the effectiveness of the tunicamycin treatment on N-linked glycosylation. Also consistent with O linkage, treatment of AEV-infected cell lysates with 50 mM NaOH at 45°C for 15 h abolished the ability of the v-*erbA* protein to bind to WGA-Sepharose (Fig. 5B, lanes 1 and 2). However, significant protein degradation was also detected after alkali treatment (Fig. 5B, lanes 3 and 4), making the results of this last assay somewhat more difficult to interpret.

In a number of my experiments, I have observed a doublet of low-abundance proteins with an apparent molecular weight of approximately 45,000 to 50,000 that also appears to bind to the WGA-Sepharose and is detected by anti-*erbA* but not by preimmune or anti-virus *gag* antiserum (data not shown). This doublet is a possible candidate for the product of the chicken c-*erbA* gene (1); however, because of the relatively low level of expression of these polypeptides, confirmatory analyses have not yet been performed.

Conclusions. My observations indicate that a small subpopulation of the v-*erbA* protein, estimated as 2 to 5% of the total, is glycosylated in AEV-infected cells. This glycosyl-

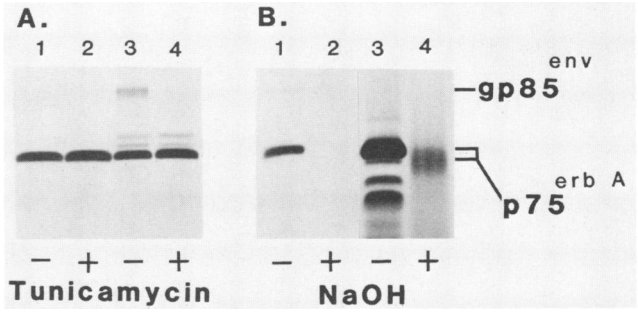

FIG. 5. Characterization of the glycosyl linkage in v-*erbA* protein. (A) Effect of tunicamycin. AEV-infected erythroid cells were radiolabeled with ^{35}S-amino acids either in the presence (+) or absence (−) of tunicamycin. The cells were lysed, and the ability of the v-*erbA* protein to bind to WGA-Sepharose was determined as described in the legend to Fig. 1. Proteins bound to the immobilized matrix were analyzed with either anti-*erbA* (lanes 1 and 2) or anti-virus (lanes 3 and 4) serum. (B) Sonic extracts of ^{35}S-amino acid-labeled, AEV-infected erythroid cells were treated with mild alkali (see text; lanes 2 and 4) or not treated (lanes 1 and 3). The lysates were subsequently incubated with WGA-Sepharose, and the proteins bound (lanes 1 and 2) or not bound (lanes 3 and 4) by the lectin were immunoprecipitated by anti-*erbA* serum and analyzed by SDS-polyacrylamide gel electrophoresis. Lanes 1 and 2 were exposed 20 times longer than lanes 3 and 4.

ation is resistant to tunicamycin treatment, suggesting that the glycosyl moieties may be in O linkage to the polypeptide chain. The carbohydrate moieties appear to be added to the nascent v-*erbA* protein within the first 1 to 2 h after synthesis, and the v-*erbA* protein chains in the nucleus appear to be more heavily glycosylated than those in the cytoplasm. Thus, the v-*erbA* protein joins the family of transcription factors and their derivatives which bear glycosyl moieties. The functional significance of transcription factor glycosylation, however, remains to be fully established.

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