Intracellular Precursors to the Major Glycoprotein of Avian Oncoviruses in Chicken Embryo Fibroblasts

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A 96,000-dalton glycoprotein, p(96), was present in cell extracts obtained from gs-chf" chicken embryo fibroblasts infected with the avian RNA tumor viruses Rous-associated virus-2 subgroup B (RAV-2) and the Schmidt-Ruppin strain of Rous sarcoma virus subgroup A (SR-RSV-A), as well as from uninfected gs,chf" (Hg) cell extracts. It was not found in cell extracts from uninfected gs-chf" or gs-chf" (Hh) cells, nor from gs-chf" cells infected with envelope-deficient Bryan high-titer Rous sarcoma virus. Immunoprecipitation, kinetic, and biochemical data indicate that this polyprotein contains information that gives rise to the major virion glycoprotein gp85. A second polyprotein of 80,000 daltons, p(80), is also present in the RAV-2- and SR-RSV-A-infected gs-chf" cells. This second polyprotein contains less carbohydrate than p(96), and kinetic and biochemical data indicate that p(80) may be an immature form of p(96).

The biosynthesis of both avian and murine RNA tumor virus structural proteins in virus-infected tissue culture cells has been actively studied in recent years. Polypeptide precursors to the internal structural virion proteins have been detected in the avian (20, 21) and murine (11, 17, 19) systems. Similarly, larger polypeptide precursors to the virus glycoproteins have been specifically immunoprecipitated from the cytoplasm of Rauscher murine leukemia virus-infected cells (1, 4; D. L. Buchhagen, Ph.D. thesis, Cornell University, Ithaca, N.Y., 1975), and several reports have described presumptive precursors to avian RNA tumor virus glycoproteins (3, 6). This report describes the finding of a glycoprotein with an apparent molecular weight of 96,000 daltons that serves as a precursor to the major avian viral envelope glycoprotein (gp85). The detailed immunological and biochemical characterizations of this precursor protein are presented here as well as evidence suggesting its derivation from an 80,000-dalton glycoprotein. Also presented are kinetic data relating to the synthesis and disappearance of this precursor in the cytoplasm of virus-infected cells pulsed with radioactive amino acids and the appearance of the radiolabel in the gp85 of intact extracellular virions.

MATERIALS AND METHODS

Cells and viruses. Cells used for the propagation of viruses were prepared as primaries from embryonated eggs supplied by SPAFAS, Norwich, Conn., and were grown in complete Scherer medium containing 5% calf serum. Only cells that typed as gs-chf" by complement fixation tests and helper assays (8) were used for virus propagation. Some uninfected cells that typed as gs-chf" (Hg) and gs,chf" (Hh) were used in designated experiments (8).

Viruses used were the Schmidt-Ruppin strain of Rous sarcoma virus subgroup A (SR-RSV-A), Rous-associated virus-2 subgroup B (RAV-2), and envelope-deficient Bryan high-titer Rous sarcoma virus [BH-RSV(→)] (7). Secondary cultures of cells were infected by the addition of 0.1 ml of stock virus solution to subconfluent monolayers of cells in 100-mm-diameter plastic petri dishes (Lux Plastics).

Antiserum. Rabbit antiserum was prepared by J. H. Chen using RAV-60 gp85 that had been purified by chromatography on Bio-Gel A-5m agarose in guanidine hydrochloride-containing buffer (5). Rabbits were hyperimmunized by one injection of 400 µg of purified RAV-60 gp85 in complete Freund adjuvant, followed by two booster injections of 200 µg of protein each in incomplete Freund adjuvant at 4 and 6 weeks after the initial injection. A precipitating antiserum was prepared by D. L. Buchhagen and G. Notani by immunizing a goat twice with 100 µg of purified rabbit immunoglobulin G in complete Freund adjuvant.

Radiolabeling of viruses and cells. Whole radiolabeled virus was prepared by labeling infected cells with 1,4C-amino acids (New England Nuclear) at 25 µCi in 5 ml of Scherer medium containing 5% calf serum and lacking tryptose phosphate broth for two 16-h periods, followed by a 16-h chase using Scherer medium containing both 5% calf serum and tryptase phosphate broth (complete Scherer medium).

 Cultures were pulsed for the times indicated by the addition of 25 µCi of 1,4C-labeled l-amino acids in 1.0 ml of Earle balanced salt solution to the infected cells in 100-mm-diameter plates; chases were performed by replacing the labeling medium with 10 ml of complete Scherer medium. Pulses and chases were terminated by washing the monolayers twice with 10 ml of cold

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phosphate-buffered saline. Labeled sugars were used in the same manner at the following concentrations: D-[\(^{14}\)C]mannose (10 \(\mu\)Ci/ml); D-[\(^{14}\)C]glucosamine (10 \(\mu\)Ci/ml); D-[\(^{3}\)H]galactose (200 \(\mu\)Ci/ml); and L-[\(^{3}\)H]fructose (200 \(\mu\)Ci/ml).

**Inhibitors.** D-Glucosamine hydrochloride (Matheson, Coleman, and Bell) or 2-deoxy-d-glucose (Calbiochem) was added to complete Scherer medium at a concentration of 10 mM.

**Virus purification.** Radioabeled virus was purified as outlined previously (18) by pelleting the virus from culture supernatants through 20% sucrose in 0.01 M Tris (pH 7.4)-0.1 M NaCl-0.001 M NaEDTA (TNE) onto a cushion of 60% sucrose-TNE in an SW27.1 rotor at 119,000 \(\times\) g for 3 h at 4°C. The cushioned virus was then banded in 15 to 50% sucrose-TNE gradients in an SW27.1 rotor at 119,000 \(\times\) g for 16 h at 4°C. Banded virus was pelleted in a type 40 rotor at 111,000 \(\times\) g for 90 min at 4°C, and virus pellets were kept frozen at −70°C until needed.

**Immuno precipitation.** Confluent monolayers of chicken embryo fibroblasts were labeled with the radioactive precursor according to the particular experiment, and cytoplasmic extracts were prepared as described by Vogt and Eisenman (20) by the addition of a buffer containing 0.5% Nonidet P-40 and 0.5% sodium deoxycholate. To 200 \(\mu\)g of total cellular protein per reaction was added the monospecific antiserum at a final dilution of 1:40. This mixture was incubated at 37°C for 1 h and at 4°C for 16 h. Precipitating antiserum was then added at a 5:1 ratio to the first antiserum; the reaction mixtures were incubated at 37°C for 15 min and then at 4°C for 4 h. Immune precipitates were collected by centrifugation in a Sorvall RC2-B centrifuge using an SE-12 rotor at 10,000 \(\times\) g for 15 min at 4°C.

**SDS-PAGE.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as follows. Gradient polyacrylamide slab gels (7.5 to 15%) were prepared using modifications of Laemmli (14). Separating gels were 13.0 cm long and 1.25 mm thick; 4.5% stacking gels were 1.5 cm long. Samples were solubilized in solutions having the following final concentrations: 0.0625 M Tris-hydrochloride (pH 6.8), 2% SDS (Matheson, Coleman, and Bell), 0.025 M dithiothreitol, 10% glycerol, and 0.01% bromophenol blue. Gels were run at 20 mA constant current and were fixed and stained with 0.25% Coomasie brilliant blue in 45% methanol-10% acetic acid. Progressive destaining was in 45% methanol-10% acetic acid and then in 7.5% methanol-5% acetic acid. Gels were impregnated with PPO (2,5-diphenyloxazole) according to the procedure of Bonner and Laskey (2) and dried. Autoradiography was carried out with Kodak RP Royal X-Omat medical X-ray film at −70°C; films were developed in a Kodak Rapid-Process automatic developing unit.

**RESULTS**

**Immunoprecipitation of proteins from the cytoplasm of infected cells.** Rabbit anti-RAV-60 gp85 serum was used to precipitate pulse-labeled proteins from the cytoplasm of chicken embryo fibroblasts that had been infected with RAV-2 and SR-RSV-A viruses (Fig. 1, lanes b and c). The major protein that was precipitated migrated in this SDS-PAGE slab gel system with an apparent molecular weight of 96,000 and is designated p(96). A second protein, p(80), of approximately 80,000 daltons, was often precipitated by the anti-gp85 serum; however, the quantity of this protein in the immune precipitates was highly variable from one experiment to the next, although it was present in

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**Fig. 1.** SDS-PAGE analysis of immunoprecipitates of pulse-labeled chicken embryo fibroblast cytoplasm. Cytoplasmic extracts of gs\({}^{chf}\) cells were prepared after a 20-min pulse with L\(^{14}\)C-amino acids. Portions of the extracts were reacted with rabbit anti-RAV-60 gp85 serum, and the resulting precipitates were subjected to electrophoresis in 7.5 to 15% gradient slab gels. Electrophoresis was carried out at 20 mA per gel (constant current). The following gs\({}^{chf}\) cell extracts were used: lane a, uninfected; lane b, RAV-2-infected; lane c, SR-RSV-A-infected; and lane d, BH-RSV(−)-infected.
largest quantities in lysates from cells infected with RAV-2 virus. p(96) was not detected in immune precipitates obtained by using either uninfected gs\(^{-}\)chf\(^{-}\) cells (lane a) or BH-RSV(\(-\))-infected gs\(^{-}\)chf\(^{-}\) cells (lane d). Further, neither p(96) nor p(80) was precipitated by rat antisera prepared against purified proteins p27, p19, and p15 derived from RAV-2 (D. L. Buchhagen, unpublished data). Conversely, the specificity of the rabbit anti-gp85 serum was further established by its failure to precipitate the major virion proteins p27, p19, and p15. Many protein bands, including those of approximately 96,000 and 80,000 daltons, were detectable in all immune precipitates including those obtained from uninfected gs\(^{-}\)chf\(^{-}\) cells and were assumed to be comigrating host proteins. The dark band at approximately 40,000 to 50,000 daltons is an artifact produced by the presence of large amounts of immunoglobulins in the precipitates.

Presence of p(96) in cells having different levels of helper activity. Uninfected chicken embryo fibroblasts that were either gs\(^{+}\)chf\(^{+}\), gs\(^{+}\)chf\(^{+}\) (H\(_{1}\)), or gs\(_{1}\)chf\(^{+}\) (H\(_{2}\)) were pulse-labeled with radioactive amino acids for 20 min, and immune precipitates were prepared as described previously, by using equal quantities of total protein from each cell lysate. p(96) was detectable at low levels in extracts obtained from pulsed gs\(_{1}\)chf\(^{+}\) (H\(_{2}\)) cells (Fig. 2, lanes e and f), but not in those from gs\(^{-}\)chf\(^{-}\) (lane b) or from gs\(^{+}\)chf\(^{+}\) (H\(_{1}\)) cells (lanes c and d). The p(96) from the gs\(_{1}\)chf\(^{+}\) (H\(_{2}\)) cells comigrated with that obtained from RAV-2-infected gs\(^{-}\)chf\(^{-}\) cells (lane a). Only immune precipitates from RAV-2-infected cultures (lane a) had demonstrable p(80). The failure to detect either p(80) in any uninfected cell lysates or p(96) in gs\(^{-}\)chf\(^{-}\) (H\(_{1}\)) cells may be due to the insensitivity of the detection method chosen or may be due to a real absence of the proteins studied.

Evidence that both p(96) and p(80) are glycosylated. To determine whether the proteins p(96) and p(80) that are specifically recognized by an antiserum to a virion structural glycoprotein are themselves glycosylated, cells infected with RAV-2 were pulsed for 20 min with either \(\text{D-[^{14}C]mannose}\), \(\text{D-[^{14}C]glucosamine}\), \(\text{D-[^{3}H]galactose}\), or \(\text{L-[^{3}H]fucose}\) (Fig. 3) as described in Materials and Methods. Under

![Fig. 2. SDS-PAGE analysis of immune precipitates prepared as described in the legend to Fig. 1. Lane a, RAV-2-infected gs\(^{-}\)chf\(^{-}\); lane b, uninfected gs\(^{-}\)chf\(^{-}\); lane c, gs\(^{-}\)chf\(^{+}\) (H\(_{2}\)); embryo 3006; lane d, gs\(^{-}\)chf\(^{+}\) (H\(_{2}\)); embryo 3010; lane e, gs\(_{1}\)chf\(^{+}\) (H\(_{2}\)); embryo 3007; lane f, gs\(_{1}\)chf\(^{+}\) (H\(_{2}\)); embryo 9838; lane g, purified \(\text{L-[^{14}C]amino acid-labeled RAV-2 virus}\).](http://jvi.asm.org/)
viruses (4, 9, 12, 13). It should be noted that cell morphology was normal after the 8-h pretreatment, and the patterns of labeled cellular proteins nonspecifically precipitated in the immune complexes were similar for both treated and untreated cultures.

**Kinetics of synthesis of p(80), p(96), and gp85.** A series of immune precipitates obtained from RAV-2-infected cultures that had been pulsed with L-^{14}C-amino acids for periods ranging from 2 to 20 min, or pulsed for 20 min and then chased with complete medium lacking the label for 1 to 4 h, are depicted in Fig. 5. Only

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**Fig. 3.** Immune precipitates of extracts from cells labeled for 20 min each as follows: lane a, D-[^14]C/mannose; lane b, D-[^14]C/glucosamine; lane c, D[^3]H/galactose; lane d, L[^3]H/fucose.

In these conditions, both p(96) and p(80) could be labeled with mannose (lane a) and glucosamine (lane b); galactose (lane c) could be detected only in p(96), and fucose (lane d) could not be detected in either protein.

**Sensitivity of the synthesis of p(96) and p(80) to known inhibitors of glycosylation.** Pretreatment of virus-infected cells for 8 h with either 2-deoxy-D-glucose or D-glucosamine hydrochloride at concentrations of 10 mM substantially decreased the subsequent 20-min incorporation of L-^{14}C-amino acids into p(96) and p(80) as shown in Fig. 4 (lanes b and c). This finding is consistent with the known effects of these drugs on the maturation of various enveloped

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**Fig. 4.** Immune precipitates of extracts from RAV-2-infected gs^-ch^- cells that had been pretreated for 8 h with 10 mM concentrations of inhibitors before receiving 20-min pulses of L-^{14}C-amino acids. Cell extracts: lane a, untreated; lane b, 2-deoxy-D-glucose-treated; lane c, D-glucosamine hydrochloride-treated.
p(80) could be detected during a 2-min pulse (lane a), whereas both p(80) and p(96) were detected after a 5-min or longer pulse (lanes b–e). Likewise, p(80) almost entirely disappeared from the cytoplasm within 1 h after a 20-min pulse (lane f), but the level of p(96) diminished more gradually beginning 2 h into the chase period. The protein bands designated y and z represent stable proteins that are present in all immune precipitates and most probably are host proteins. The assignment of protein x to either cell or virus was more difficult since this band seemed to diminish rapidly after a 20-min pulse, although a residual band was detectable after longer chase periods. These data can be compared with densitometer tracings of a gel on which viruses harvested from the supernatants of the chased cultures were subjected to electrophoresis (Fig. 6, a–e). It is readily apparent that the major internal virion proteins are fully represented in the harvested viruses as early as 1 h after the pulse; however, gp85 did not become detectable in mature virions until 3 h after the pulse (Fig. 6c). These kinetic studies are summarized in Fig. 7. The 2-h lag between the disappearance of 50% of the cytoplasmic p(96) label present in the 20-min pulsed-and-chased samples and the appearance of 50% of the final incorporation level of label in the gp85 of mature virions should be noted.

**DISCUSSION**

The presence in virus-infected cells of a polyprotein precursor to the avian virion glycoprotein gp85 represents the identification of a second major class of precursors to the virion structural proteins. The precursor to the internal virion proteins, pr76, contains information which eventually gives rise to p27, p19, p15, and p12 proteins (21). The immunoprecipitation, kinetic, and biochemical data presented here confirm that a glycosylated polyprotein of 96,000 daltons eventually matures to a smaller glycoprotein of 85,000 daltons. This p(96) most likely corresponds to the p90 polyprotein reported by England et al. (3) and the gp92 of Moelling and Hayami (15).

A second immune precipitated protein, p(80), appears in, and chases from, immune precipitates prior to p(96); thus, p(80) may represent an immature form of the glycoprotein precursor p(96) and may be analogous to the putative

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**FIG. 5.** Immune precipitates from RAV-2-infected gs’chf’ cells that were pulsed with 1.14C-amino acids. Pulse times: lane a, 2 min; lane b, 5 min; lane c, 10 min; lane d, 15 min; lane e, 20 min; lane f, 20-min pulse, 1-h chase; lane g, 20-min pulse, 2-h chase; lane h, 20-min pulse, 3-h chase; lane i, 20-min pulse, 4-h chase; lane j, 1.14C-amino acid-labeled RAV-2 virus.
glycoprotein precursor p70 described by Halpern et al. (6). Further, both p(80) and p(96) contain glucosamine and mannose, two sugars that are often present in core carbohydrate structures attached to protein backbones through glucosamine linkages to asparagine residues. The detection of galactose only in p(96) may indicate that p(96) is more mature than p(80) with regard to glycosylation, although the presence of this sugar in a much lower quantity in p(80) cannot be ruled out. The inhibition of the synthesis of p(96) and p(80) by D-glucosamine, in contrast to an accumulation of a smaller, nonglycosylated protein that is not cleaved, suggests that the addition of the carbohydrate residues occurs during translation and may be requisite for its continuation. Hayman et al. (9) had determined that pr76 synthesis occurred, but that cleavage was greatly reduced under a similar glucosamine block.

No intracellular gp85 was detected in these investigations, although England et al. (3) could demonstrate that their presumptive precursor chased to intracellular gp85. Our failure to detect the mature viral glycoprotein in the cytoplasmic extracts can be explained in any of several ways. One possibility is that the embryos used to prepare the monolayers for our experiments came from a different flock of chickens than those used by the other investigators and may have a different or altered pathway for glycoprotein precursor processing. Another possibility is that gp85 may be very firmly associated with the cellular plasma membrane and is not released by the addition of detergents; it would then be lost in the material pelleted during the preparation of the cytoplasmic extracts. The 2-h lag that was observed between the disappearance of 50% of the labeled cytoplasmic p(96) and the appearance of labeled gp85 in virions at 50% maximal level suggests that there may be sequestration of the p(96) in a membrane fraction. Such a phenomenon has been reported for the murine glycoprotein precursor (22), and it is possible that the subsequent maturation steps include specific proteolytic cleavages. These later steps in virus maturation are the subject of our current investigations. It also should be pointed out that, contrary to the findings of
others (3), no gp37 was detected in immune precipitates obtained from cytoplasmic extracts by using the anti-gp85 serum. This failure may be explained by the same possibilities raised with regard to the absence of gp85 from the cytoplasm of the infected cells or may be due to a lack of immunoreactive sequences between gp85 and gp37, as has been suggested by Mosser et al. (16).

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LITERATURE CITED