KEY WORDS:avian endogenous/retroviruses/envelope/glycoprotein

Characterization of the Endogenous Retroviral Envelope Glycoproteins Found in the Sera of $ev_3$ and $ev_6$ Chickens

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(Accepted 23 May 1985)

SUMMARY

Chicken sera may contain components that immunologically crossreact with avian retroviral glycoprotein. Sera from chickens carrying the endogenous viral loci $ev_3$ and $ev_6$ contain glycoproteins with molecular weights of approx. 85000, which on the basis of tryptic peptide analysis are related to exogenous viral glycoprotein. Such endogenous viral glycoproteins are present in a free form, lacking disulphide-bonded gp35.

Endogenous retroviruses have been detected in a large number of animal species and have been particularly well studied in the domestic chicken, Gallus gallus, where some 20 different loci ($ev$ loci) have been defined (Astrin, 1978; Astrin et al., 1980; Rovigatti & Astrin, 1983; Humphries et al., 1984). Individual chickens vary in the numbers and combinations of endogenous viruses integrated in their genomes (Hughes et al., 1981) and it has been possible to establish which $ev$ loci are responsible for particular phenotypes of expression. For example, the presence of endogenous viral glycoproteins in fibroblasts, previously determined in the 'chicken helper factor (chf) assay' (Hanafusa et al., 1973), results from expression of the $ev$ loci 3, 6 or 9 (Baker et al., 1981; Rovigatti & Astrin, 1983).

In previous studies, using immunological methods, we demonstrated that material immunologically crossreacting with viral glycoprotein was present in the sera of certain uninfected chickens (Bosch et al., 1978, 1983). In the present paper we have characterized the crossreacting antigen in chicken serum in order to establish that it is truly endogenous viral glycoprotein and to determine its molecular nature.

Sera from chickens with the following genotypes were employed: $ev_1$, 2, $ev_1$, 3, and $ev_1$, 6 (referred to as $ev_2$, $ev_3$ and $ev_6$ sera). In addition, a serum from a chicken lacking $ev$ loci (Astrin et al., 1979) was employed ($ev^-$). The sera were generously supplied by Dr D. L. Ewert, Wistar Institute, Philadelphia, Pa., U.S.A. Using a radioimmunoassay as described previously (Bosch et al., 1978) employing purified $^{125}$I-gp85 from Prague C Rous sarcoma virus (RSV) and anti-Prague B RSV serum it could be established that the sera from the $ev_3$ and $ev_6$ animals contained material crossreacting with viral glycoprotein, whereas the sera from the $ev_2$ and $ev^-$ animals were completely negative. The $ev_3$ and $ev_6$ sera contained approximately equal amounts of crossreacting material, estimated to be 1 to 2 μg crossreacting antigen per ml serum.

As a first step in characterizing the crossreacting material in serum, different chicken sera were analysed by immunoblotting (Western blotting). In order to increase the sensitivity and reduce the background, aliquots of chicken serum were first immunoprecipitated with an antiserum prepared against Prague B RSV, and the immunoprecipitates were subjected to gel electrophoresis. After electroblotting, the transferred proteins were analysed using an antiserum specific for the viral glycoprotein gp85. In Fig. 1(a), as a control purified Prague C RSV was electrophoresed under reducing conditions and a single band in the position of gp85 was observed as expected. In lanes (b) and (c), immunoprecipitates from $ev_2$ and $ev^-$ sera were

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Short communication

Fig. 1. Immunoblot of endogenous viral glycoprotein in ev-defined chicken sera. Washed immunoprecipitates generated as described below were electrophoresed on a 9% slab gel under reducing conditions with mercaptoethanol (a to f) or non-reducing conditions (g to k). After electroblotting onto nitrocellulose paper, lanes (a) to (j) were reacted with monospecific anti-gp85 serum (Bosch & Schwarz, 1984) and lane (k) with normal rabbit serum, followed by 125I-Protein A. The immunoprecipitates were obtained from chicken serum (30 µl) or purified Prague C RSV (1 µg) by reaction with anti-Prague B RSV serum (a to e and g to k) or with normal rabbit serum (f). The materials immunoprecipitated were (a) Prague C RSV, (b) ev2 serum, (c) ev-serum, (d) ev6 serum, (e,f) ev3 serum, (g) ev6 serum, (h) ev3 serum, (i) ev-serum, (j, k) Prague C RSV. VGP, position of the disulphide-linked viral glycoprotein complex.

electrophoresed, i.e. sera from chickens which do not express endogenous glycoproteins. Two weak non-specific bands can be observed. The smaller component with a molecular weight of approximately 20000 was also observed after immunoprecipitation with normal rabbit serum. The higher mol. wt. band of approximately 75000 is probably chicken IgG heavy chain which can be recognized by its carbohydrate determinants shared with the viral glycoprotein. In lanes (d) and (e), immunoprecipitates from ev6 and ev3 sera, respectively, were electrophoresed and in each case a specific band with a mol. wt. approximately the same as gp85 (ev3) or slightly higher (ev6) was observed. These presumably represent the endogenous viral glycoprotein which competes in the radioimmunoassay. That the ev6 glycoprotein behaved as if it were larger than the ev3 viral glycoprotein was confirmed in experiments with several different ev3 and ev6 animals. Furthermore, analysis of the serum from a chicken containing both ev3 and ev6 loci resulted in the appearance of a double band following Western blotting (data not shown). That the specific bands from ev3 and especially ev6 sera do not exactly co-migrate with Prague C gp85 is not surprising in view of the fact that the large glycoproteins from different subgroups of RSV also differ in their molecular weights. In order to establish that the 85000 mol. wt. bands seen in ev3 and ev6 sera represent endogenous viral glycoproteins, immunoprecipitates were digested before electrophoresis with endoglucosidase H, which removes high-mannose oligosaccharides. This resulted in a reduction of the mol. wt. (data not shown), and indicates that the band contained a glycoprotein consistent with the established fact that approximately 30% of the oligosaccharides on the viral glycoprotein are of the high-mannose or hybrid type (Hunt & Wright, 1981; Bosch et al., 1982; Bosch & Schwarz, 1984).

The tryptic peptide map of the iodinated gp85 from the Schmidt–Ruppin B strain of RSV was compared to that of the specific band from ev3 serum (Fig. 2). It was seen that a number of major spots are shared by the two components, thus unequivocally showing that the band seen after Western blot analysis is related to gp85 and represents endogenous viral glycoprotein.
Fig. 2. Tryptic peptide maps of endogenous viral gp85 from ev 3 chicken serum (a,a') and exogenous viral gp85 from Schmidt–Ruppin B RSV (b,b'). Specific components were purified and iodinated as follows. Five ml ev 3 serum or 500 gg purified Schmidt–Ruppin B-RSV was immunoprecipitated with monospecific anti-gp85 serum and the immunoprecipitates were electrophoresed. The position of viral glycoprotein was determined by electrophoresis of \(^{125}\text{I}\)-gp85 from Prague C RSV on the same slab gel. The appropriate regions of the gel were cut out, proteins eluted, lyophilized and subsequently radioiodinated using the chloramine-T method. The iodinated samples were re-immunoprecipitated with anti-gp85 serum followed by Protein A. The immunoprecipitates were separated from the bacteria with 2\% SDS and precipitated with acetone. Tryptic peptides were prepared from this material and separated by two-dimensional analysis on thin-layer cellulose sheets as described (Gebhardt et al., 1984). The spots were visualized by autoradiography. In the schematic drawings shown in (a') and (b') the shaded spots represent shared peptides. S indicates the origin.

Similar results were obtained for the component from ev 6 serum (not shown) confirming that this too represents endogenous viral glycoprotein.

In Fig. 1, the antiserum employed was specific for gp85. In mature virus, gp85 is disulphide-linked to the smaller glycoprotein gp35 to form the viral glycoprotein complex (Leamnson & Halpern, 1976; Pauli et al., 1978). It was thus of interest to determine whether the endogenous viral gp85 observed in Fig. 1 was present as a complex with gp35. We have prepared an
antiserum specific for viral gp35 (Bosch & Schwarz, 1984) but although this antiserum can specifically precipitate gp35, it was not reactive following immunoblotting. When electrophoresis was performed under non-reducing conditions, the viral glycoprotein complex remained intact and migrated with an approx. mol. wt. of 120000. In Fig. 1(k) this can be seen for Prague C-RSV. In lanes (h) and (g) the immunoprecipitates from ev3 and ev6 sera were electrophoresed under non-reducing conditions. In each case there was no shift to a higher mol. wt., indicating that the endogenous viral gp85 in chicken serum is not present as a disulphide-bonded complex with endogenous gp35. In the case of ev3, the specific band migrated identically under reducing and non-reducing conditions (lanes e and h), whereas the ev6 band migrated even faster under non-reducing conditions (lanes d and g). This may be due to the ev6 glycoprotein having a different conformation under non-reducing as compared to reducing conditions; a similar phenomenon has previously been observed with the E2 glycoprotein of Semliki Forest virus (Kaluz & Pauli, 1980).

In order to confirm that serum endogenous gp85 is not present in a viral glycoprotein complex we examined the ability of several antisera to remove the specific competing material. Chicken sera were preincubated with either anti-gp85, anti-gp35 or normal rabbit serum, immune complexes were precipitated with Protein A-carrying bacteria, and the supernatants were tested in a radioimmunoassay for the presence of a competing activity. In Fig. 3(b), a control analysis using a lysate of purified Schmidt-Ruppin A RSV is shown. In this case gp85 and gp35 were disulphide-linked so that the competing activity, namely gp85, could be removed by preincubation with either anti-gp85 or anti-gp35 sera. Preincubation with normal rabbit serum had no effect. In Fig. 3(a), the same experiment was performed with ev3 serum. In this case anti-gp85 serum removed the competing activity, but anti-gp35 serum could not, again illustrating that the endogenous viral gp85 is not disulphide-linked to form a viral glycoprotein complex. Similar results were obtained with ev6 serum (data not shown). Since it was possible that viral glycoprotein in chicken serum had undergone spontaneous reduction on storage, freshly obtained blood was immediately adjusted to 0-05 M-iodoacetamide which has been reported to stabilize disulphide bonds (Leamnson & Halpern, 1976). This treatment did not alter the result that gp85 is present in a uncomplexed form in chicken serum. The monospecific anti-gp35 serum used here did not react with gp35 which had been electrophoblotted. This phenomenon, which can be observed with many antisera, is presumably due to the antigen adopting a conformation on the nitrocellulose paper which can no longer be recognized by antibody. However, for this reason, it has not been possible to determine whether endogenous viral gp35 is present in chicken serum in a form not complexed to gp85.
RSV gp35 is a transmembrane glycoprotein (Hunter et al., 1983; Schwartz et al., 1983) whereas gp85 is held onto the virus through disulphide-bonding to gp35 (Leamnson & Halpern, 1976; Pauli et al., 1978). It has been demonstrated that in cultured fibroblasts endogenous viral glycoprotein is also present in the form of a disulphide-linked complex (VGP) with a proportion being in the form of two VGP complexes disulphide-linked to one another (VGP₂) (Ewert & Halpern, 1982a). Since endogenous serum gp85 is in a free form, it is possible that it has arisen by reduction of VGP molecules at the surface of those cells which produce it. In this case gp35 would remain embedded in the cell membrane. On the other hand, it is possible that the cells which produce endogenous serum gp85 secrete the viral glycoprotein complex as such and reduction to yield free gp85 occurs subsequently. In this case, gp35 would also be present in serum, possibly in the form of aggregates since gp35 molecules are very hydrophobic. At the moment, there is no way to differentiate between these two alternatives. The question as to which cells produce the endogenous gp85 found in serum can also not be answered at present. It has, however, been demonstrated that chf⁺ cells of the lymphocyte lineage are positive for the expression of envelope antigen (Ewert & Halpern, 1982b; Ewert et al., 1983, 1984), and it is tempting to postulate that these cells are in part responsible for the presence of endogenous gp85 in serum. Finally, the possible function of endogenous gp85 in serum also remains obscure. The fact that chickens completely lacking endogenous viruses (ev⁻) exist and are fertile indicates that endogenous viruses are not essential. It is, however, possible that endogenous viral glycoprotein in chicken serum as demonstrated here or in mouse serum (Hino et al., 1976) do play more subtle roles hitherto not determined.

We should like to thank H. Bauer, R. R. Friis, and A. Ziemiecki for useful discussions. We are grateful to Dr D. L. Ewert for sera of ev⁻-defined chickens. This work was supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 47, Virology).

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Short communication


*(Received 15 January 1985)*