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Isolation of a 48-kilodalton chicken embryo erythrocyte membrane glycoprotein by ion-exchange and gel fast protein liquid chromatography

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Membrane glycoproteins are thought to play a major role in the maturation and functions of erythrocytes[1]. During the past few years, a large number of erythrocyte membrane glycoproteins have been identified[2]. One of these, of MW 48 000, was observed on chicken embryo erythrocytes, fibroblasts and lymphocytes[3–6]. This glycoprotein disappears during cell maturation of adult chicken erythrocytes[7], and is also thought to act as a cell surface receptor for Sindbis virus haemagglutinin[8]. Its p*I* (5–6.6) and peptide mapping have been reported previously[9,10].

At this stage very little is known about the structure and the function of this glycoprotein. The aim of this study was to isolate it from surface-labelled erythrocyte membranes using ion-exchange chromatography in tandem with gel fast protein liquid chromatography (FPLC). The isolated glycoprotein was identified by its apparent molecular mass, its surface labelling and its peptide mapping characteristics. The results obtained indicate that this glycoprotein can be rapidly isolated in high purity using the FPLC system.

MATERIALS AND METHODS

Fertilized White Leghorn eggs were obtained from Spafas. Co. (Norwich, CT, U.S.A.). Whole blood was collected from 18-days-old chicken embryos by cutting open the main blood vessels and allowing the cells to be pumped or to drain into 4% sodium citrate[11]. Red blood cells (RBCs) were then centrifuged at 1300 *g* for 2 min. The supernatant was discarded and the RBCs were washed three times in complete phosphate buffered saline (PBS) pH 7.4. Since the white blood cell count in the embryonic circulation is very low until hatching[12], contamination by these cells is negligible under the experimental conditions used in this study.

Preparation of radiolabelled cells

Red blood cells were surface labelled with $Na^{125}I$ using the technique described by Morisson[13]. Briefly, RBCs were pelleted and the labelling reaction was performed at room temperature with 0.3 mCi of ^{125}I + hydrogen peroxide + lactoperoxydase

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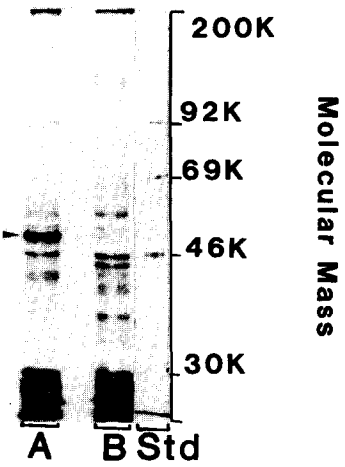

Fig. 1. Membrane preparation from 18-days-old chicken embryo erythrocytes (A) and chicken adult erythrocytes (B) were subjected to electrophoresis under reducing conditions on a 8% SDS-polyacrylamide gel. The gel was fixed in 50% methanol–15% acetic acid for 20 min and silver-stained. Lanes A and B were loaded with 10 μg of each membrane preparation. Std corresponds to the molecular-mass standard. Note: the glycoprotein (MW 48 000) disappears during cell maturation of adult chicken erythrocytes.

(1 mg/ml). The labelling reaction was stopped by addition of PBS. Intensive washes with PBS were performed until no significant radioactivity was detected in the supernatant.

Isolation of erythrocyte plasma membranes

Plasma membranes were prepared from unlabelled or surface-labelled erythrocytes following the method of Dodge *et al.*[14], with some modifications. Briefly, adult or embryo chicken erythrocytes were displaced at a concentration of 10^9 cells/ml and then resuspended in five packed cell volumes of a hypotonic buffer (10 m*M* Tris pH 8.3, 1.5 m*M* magnesium chloride, 10 m*M* potassium chloride and 5 m*M* $Na_2S_4O_6$ added as a proteolytic enzyme inhibitor). After incubation at room temperature for 30 min, the RBCs were homogenized with a tight-fitting homogenizer as reported by Chan[15]. Homogenized RBCs were then spun for 2 min at 1500 *g* to pellet the nuclei[16] (centrifugation 1). Membrane fractions present in the supernatant from the first centrifugation were subjected to a second centrifugation at 7800 *g* for 30 min. The pelleted membranes were then resuspended in a lysis buffer (50 m*M* Tris pH 7.0, 2 m*M* dithiothreitol (DTT), 1% Berol 185) at a final concentration of 2 mg/ml. The detergent-solubilized membranes were then stirred for 10 min at 4°C and centrifuged for 1 h at 160 000 *g*, following the method of Lundahl *et al.*[17].

Membrane preparations from adult and embryo chicken erythrocytes are shown in Fig. 1.

FPLC

The FPLC separations were performed on a Mono Q HR 5/5 prepacked anion-exchange column (50 mm × 5 mm I.D.), a Mono S HR 5/5 prepacked cation-

exchange column (50 mm × 5 mm I.D.) and a Superose 12 HR 10/30 gel filtration column (300 mm × 10 mm I.D.) (Pharmacia, Uppsala, Sweden). Berol 185, a non-ionic detergent, was obtained from Berol Chimie (Stenungsund, Sweden). All solutions used were degassed and the erythrocyte membrane samples were filtered through a 0.2-μm filter (Millipore).

Anion-exchange chromatography

The starting buffer was 20 m*M* ethanolamine pH 9.5, 2 m*M* DTT, 0.5% Berol and the final buffer was 20 m*M* ethanolamine pH 9.5, 2 m*M* DTT, 0.5% Berol and 1 *M* sodium chloride. To avoid light absorption by oxidized DTT, this reagent was added after degassing, immediately before the experiments[17]. Berol 185-solubilized membranes were dialysed overnight at 4°C against the starting buffer. Solubilized labelled material was centrifuged in an Eppendorf centrifuge for 5 min and then the equilibrated sample was injected onto a Mono Q (HR 5/5) anion-exchange column, via a 10-ml Superloop (Pharmacia). The linear 0–0.5 *M* sodium chloride gradient was generated over 30 min at a flow-rate of 1 ml/min.

Cation-exchange chromatography

The glycoprotein (MW 48 000)-rich fractions eluted from the anion-exchange column were injected onto a Mono S (HR 5/5) cation-exchange column. The starting buffer was 50 m*M* malonic acid pH 5.8, 2 m*M* DTT, 0.5% Berol and the final buffer was 50 m*M* malonic acid pH 5.8, 2 m*M* DTT, 0.5% Berol and 1 *M* sodium chloride. Peaks eluted from the Mono Q column were dialysed overnight at 4°C against the starting buffer and concentrated to 2 ml by ultrafiltration (Millipore). The cation-exchange chromatography was performed at a flow-rate of 1 ml/min. Under such experimental conditions the glycoprotein-rich fractions were found in the flow-through. The column was then washed with the final buffer over 10 min.

Gel filtration chromatography

Peak 1 from the Mono S flow-through was ultrafiltrated then injected onto a Superose 12 column via a 500-μl sample loop. The buffer was 50 m*M* malonic acid pH 5.8, 2 m*M* DTT, 0.5% Berol and 0.1 *M* sodium chloride. The flow-rate was 0.3 ml/min over 20 min.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

A 50-μl volume of each eluted peak fraction was mixed (1:1) with denaturing buffer containing 125 m*M* Tris pH 6.0, 4% SDS, 10% β-mercaptoethanol and 30% glycerol. The samples were heated for 5 min at 100°C and the reduced material was subjected to electrophoresis on 8% SDS-polyacrylamide gel under reducing conditions, as described by Laemmli[18]. After electrophoresis, gels were fixed with 50% methanol, 15% acetic acid for 20 min and then silver-stained as described by Ansorge[19]. Silver-stained gels were dried and subjected to an indirect autoradiography, carried out using pre-flashed X-Omat films (Kodak) and Dupont-lighting intensifying screens as described by Laskey and Mills[20]. The ^{14}C-methylated markers used were myosin (MW 200 000), phosphorylase b (92 000), albumin (69 000), ovalbumin (46 000), carbonic anhydrase (30 000) (Amersham Radiochemical Centre, Amersham, U.K.).

Peptide mapping

Experiments were performed as described by Krsmanovic *et al.*[9], with minor modifications. Briefly, the radiolabelled glycoprotein (MW 48 000), isolated from the Superose 12 column, was subjected to electrophoresis under reducing conditions on a 8% SDS-polyacrylamide gel. After electrophoresis, the gel was fixed, Coomassie-stained and then dried. The glycoprotein band was excised and subjected to partial proteolysis using the V8 protease from *Staphylococcus aureus* (Miles) (2 μg/ml). After 2 h incubation at room temperature for 2 h, the digested glycoprotein was electrophorised under reducing conditions on a 14% SDS-polyacrylamide gel. The gel was dried and then prepared for autoradiography. Control experiments were performed by direct immunoprecipitation of the glycoprotein from surface-labelled erythrocyte membranes, using a polyclonal antibody raised against whole 18-days-old embryo erythrocytes. The radiolabelled band corresponding to the glycoprotein (MW 48 000) was excised and subjected to partial proteolysis as described above.

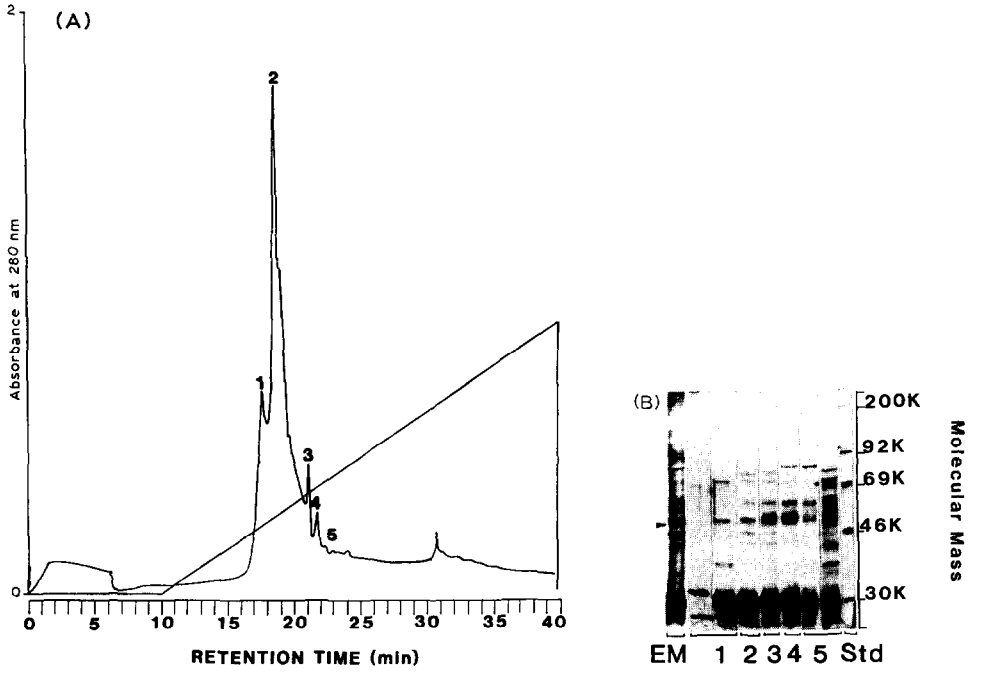

Fig. 2. (A) Chromatographic separation of radiolabelled erythrocyte membranes from an 18-days-old chicken embryo on a Mono Q column connected to a FPLC system. Erythrocyte membranes were injected on to the column via a 10-ml Superloop. The linear 0–0.5 *M* sodium chloride gradient (pH 9.5) was generated over 30 min at a flow-rate of 1 ml/min. (B) Eluted peaks were subjected to electrophoresis under reducing conditions on a 8% SDS-polyacrylamide gel, fixed and silver-stained. Lane EM corresponds to the erythrocyte embryo membrane preparation prior to the Mono Q chromatography. The numbers below the other lanes correspond to the peak numbers. Std corresponds to the molecular-mass standard.

RESULTS AND DISCUSSION

Separation of radiolabelled erythrocyte membrane glycoproteins by Mono Q anion-exchange chromatography

Using a linear 0–0.5 *M* sodium chloride gradient, five major peaks were eluted when Berol 185-solubilized erythrocyte membranes were injected onto a Mono Q column (Fig. 2A). The peaks eluted were subjected to electrophoresis under reducing conditions on a 8% SDS-polyacrylamide gel. Radiolabelled membrane glycoproteins were then observed by indirect autoradiography (results not shown) or silver staining (Fig. 2B). The glycoprotein of MW 48 000 was identified according to its molecular mass (silver staining) and its surface radiolabelling characteristics (autoradiography). In typical experiments this glycoprotein was eluted in peaks 3 and 4, between 0.15 and 0.4 *M* sodium chloride (Fig. 2B). Most of the erythrocyte proteins, as well as the glycoprotein (MW 48 000) and a predominant protein band (MW 30 000) were

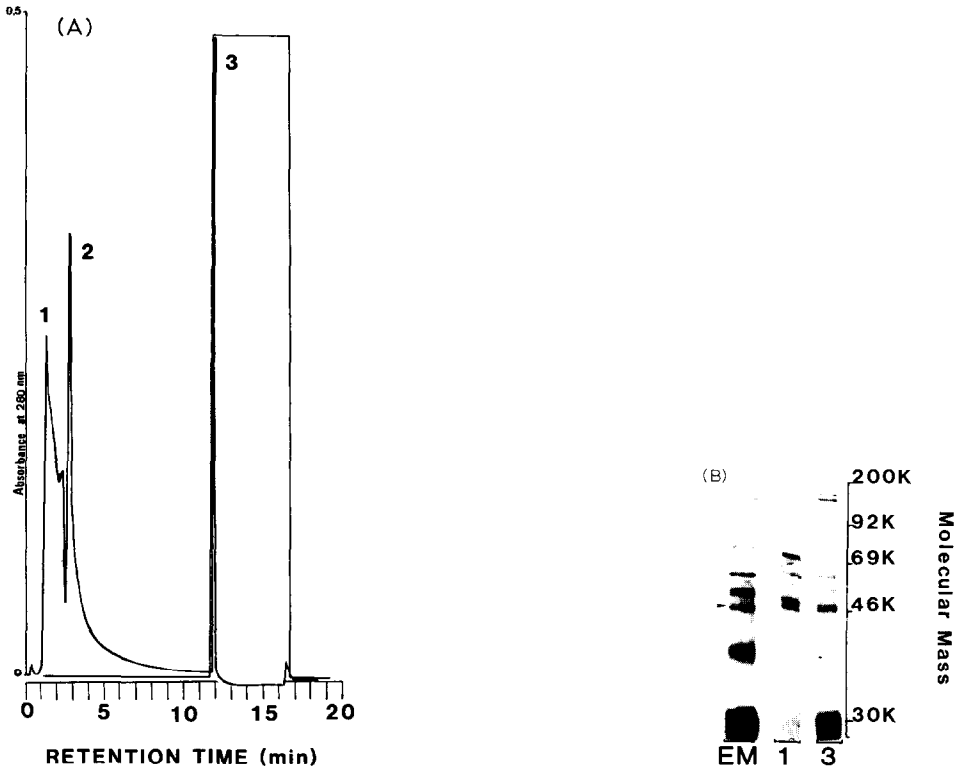

Fig. 3. (A) Isolation of the radiolabelled glycoprotein (MW 48 000) using Mono S cation-exchange chromatography. The glycoprotein-rich fractions from the Mono Q column (peaks 3 and 4) were concentrated to 2 ml and injected onto the Mono S column at a flow-rate of 1 ml/min. After collecting the flow-through (peaks 1 and 2), the Mono S column was washed for 10 min with final buffer containing 1 *M* sodium chloride (peak 3). (B) Eluted peak fractions were subjected to electrophoresis under reducing conditions on an 8% SDS-polyacrylamide gel. The gel was fixed in 50% methanol–15% acetic acid for 20 min and silver-stained. The numbers below each lane correspond to peak numbers. Lane EM corresponds to the sample prior to the Mono S chromatography, Std to the molecular-mass standard.

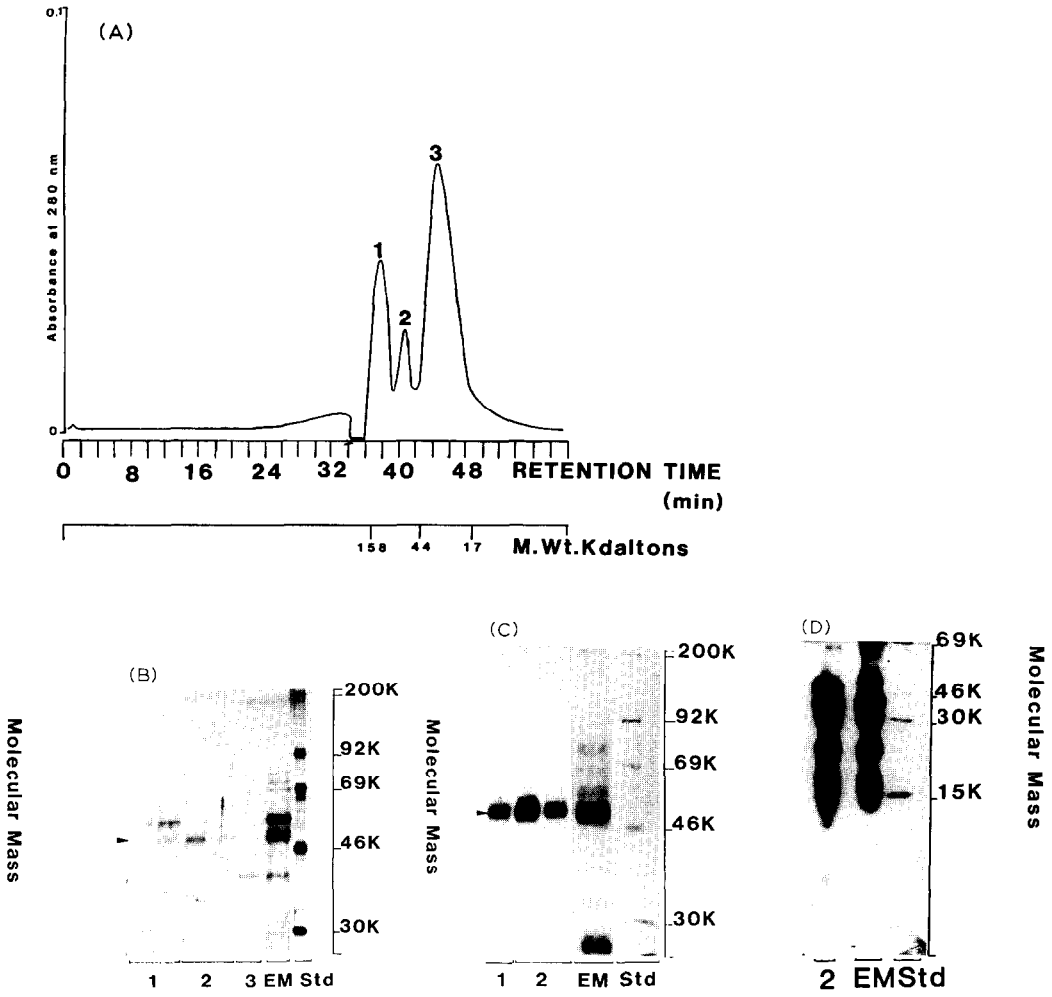

Fig. 4. (A) Further purification of the radiolabelled glycoprotein (MW 48000) by Superose 12 gel permeation chromatography. The glycoprotein isolated from the flow-through from the Mono S column (peak 1) was concentrated to 0.5 ml and injected onto the Superose 12 column at a flow-rate of 0.3 ml/min. Prior to injection, the Superose 12 column was equilibrated at a flow-rate of 0.3 ml/min with the starting buffer 50 m*M* malonic acid pH 5.8, 2 m*M* DTT, 0.5% Berol, 0.1 *M* sodium chloride. (B) Eluted peak fractions (peaks 1–3) were subjected to electrophoresis under reducing conditions on an 8% SDS-polyacrylamide gel. The gel was fixed in 50% methanol–15% acetic acid for 20 min and silver-stained. The numbers below each lane correspond to peak numbers. Lane EM corresponds to the sample prior to Superose 12 chromatography, Std to the molecular-mass standard. (C) Assessment of the purity of the isolated radiolabelled glycoprotein (MW 48000) by autoradiography. The numbers below each lane correspond to peaks eluted from the Superose 12 column. Lane EM correspoonds to the erythrocyte membranes prior the purification procedure, Std to the molecular-mass standard. Lanes 1, 2 were exposed for 48 h at −70°C. (D) Assessment of the integrity of the isolated radiolabelled glycoprotein by partial proteolysis with V_8 protease. The first lane corresponds to peak 2 eluted from the Superose 12 column. Lane EM corresponds to ^{125}I-labelled erythrocyte lysate immunoprecipitated using polyclonal antibodies raised against whole 18-days old embryo chicken erythrocytes, Std to the molecular-mass standard.

found in more than one eluted peak. It is possible that interactions between these proteins may occur, despite the presence of the non-ionic detergent (Berol 185), which would explain their elution at the same salt concentration. Similar observations were previously reported using the FPLC system[21,22]. Alternatively the presence of the component of MW 48 000 in several peaks may reflect a microheterogeneity of its carbohydrate moiety. This is consistent with the observation that this glycoprotein was separated into several isoforms, when using chromatofocusing on a Mono P column[23]. Moreover, when using electrofocusing combined with SDS-PAGE, Miller *et al.*[8] showed that the glycoprotein (MW 48 000) focused into several spots ranging in p*I* from 5 to 6.6.

Isolation of the glycoprotein (MW 48 000) using a Mono S cation-exchange column

In order to separate the main contaminants, *e.g.*, the protein band of MW 30 000, from the glycoprotein, peaks 3 and 4 eluted from the Mono Q column were pooled, concentrated and then injected onto a Mono S column. Three peaks were separated (Fig. 3A) which were subjected to SDS-PAGE on a 8% gel under reducing conditions. Labelled membrane glycoproteins in these eluted peaks were observed by indirect autoradiography (results not shown) and silver staining (Fig. 3B). By comparing the autoradiogram and the silver-stained gel, the glycoprotein (MW 48 000) was identified in peak 1 corresponding to the flow-through, whereas the predominant protein band of MW 30 000 was found mainly in peak 3 (Fig. 3B, lanes 2 and 3).

Further purification of the glycoprotein (MW 48 000) using Superose 12 gel filtration chromatography

Peak 1 eluted from the Mono S column was concentrated by ultrafiltration to 0.5 ml and injected onto a Superose 12 gel filtration column. The labelled membrane glycoproteins were resolved into three peaks, according to their molecular mass (Fig. 4A). The peaks were subjected to electrophoresis under reducing conditions on a 8% SDS-polyacrylamide gel. Silver staining (Fig. 4B) and autoradiography (Fig. 4C) of the labelled electrophorised proteins showed that peak 2 contained the glycoprotein free of any contaminants. Traces of this glycoprotein were present in peak 1 (Fig. 4B and C, lane 1). It was also identified on the basis of its peptide mapping[9]: the glycoprotein isolated by gel filtration chromatography and subjected to partial proteolysis using V8 protease shows a pattern of five different bands ranging in MW from 48 000 to 14 000 (48 000, 36 000, 30 000, 22 000, 14 000). This protelytic pattern is similar to that observed for radiolabelled glycoprotein (MW 48 000) immunoprecipitated by polyclonal antibodies (Fig. 4D).

This paper describes a method for the purification of the erythrocyte membrane glycoprotein (MW 48 000) from embryos, using FPLC. The use of erythrocyte membrane preparations instead of whole erythrocytes may allow the isolation of glycoprotein free of cytoplasmic contaminants and with a high degree of purity.

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