Isolation of a teratocarcinoma stem cell lectin implicated in intercellular adhesion

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We have previously identified a cell surface teratocarcinoma stem cell lectin with a fucan/mannan specificity. We now report the purification of the hemagglutinin (lectin) from stem cell conditioned medium by exclusion on a Sepharose 2B column, followed by elution with 0.5 M NaCl from DEAE-cellulose, providing an overall purification of about 90-fold. When this material was analyzed, by SDS-polyacrylamide gel electrophoresis, a major band of M_r 56 000 was consistently observed. Hemagglutination activity was renatured from the gels and localized exclusively to a region of the gel that, as detected by fluorography, contains only the 56-kDa component. This suggested that this polypeptide comprises the lectin.

Teratocarcinoma stem cell *Lectin* *Intercellular adhesion*

1. INTRODUCTION

Lectins were first identified in various plant species and more recently in lower eukaryotes and vertebrates, and are generally detected by their ability to agglutinate erythrocytes (hemagglutination). Although the ability to bind carbohydrate suggests a role for these proteins in recognition events, with few notable exceptions [1–3], the function of these lectins has been difficult to ascertain. We have previously detected a mannan/fucan specific cell surface lectin on teratocarcinoma stem cells based upon the ability of intact stem cells to form rosettes with specific erythrocyte types [4]. Subsequently, we identified hemagglutination activity in both aqueous and detergent extracts derived from stem cells [5,6]. This activity is virtually identical in its erythrocyte- and carbohydrate-binding specificity to the rosette-mediating activity [5]. We have also suggested that the lectin is involved in divalent-cation independent reaggregation of teratocarcinoma stem cells [4,7]. We now report the presence of the hemagglutinin (lectin) in conditioned medium from stem cells and its identification as a protein with a subunit of M_r 56000.

Abbreviations: CMF-PBS, calcium and magnesium-free Dulbecco's phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

2.1. *Cell extracts*

Teratocarcinoma stem cell line Nulli SCC-1 was cultured as in [8]. To obtain conditioned medium, cultured cells (approx. 2×10^7 cells/100 mm dish) were first washed 3 times in 5 ml of serum-free medium (Dulbecco's-modified Eagle's medium, 4.5 g/l glucose), and incubated for 16 h at 37°C in 5 ml of serum-free medium. After this incubation the cells remained spread and attached to the dish and appeared healthy. The medium was then removed from the dish, centrifuged at $500 \times g$ for 5 min to remove cell debris, and dialyzed extensively against CMF-PBS. [^{35}S]Methionine-labeled conditioned medium was prepared by addition of 50–100 μCi per dish of L-[^{35}S]methionine (Amersham Radiochemicals, spec. act. 1225 Ci/mmol) at the outset of the 16 h incubation.

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2.2. *Lectin assays and purification*

Lectin activity was assayed by a hemagglutination assay as described [5]. Specific activities were expressed either as (i) hemagglutination endpoint/mg protein as determined by the method of Lowry et al. [9] or (ii) hemagglutination endpoint/cpm of an aliquot of [^{35}S]methionine-labeled extract or conditioned medium (samples were diluted into Hydrofluor [National Diagnostics] and counted in a Beckman LS-8000 liquid scintillation counter). Gel filtration was performed in CMF-PBS using a 2.5 × 45 cm Sepharose 2B (Pharmacia) column. After gel filtration of conditioned medium, the fractions containing hemagglutination activity were pooled and Triton X-100 was added to a final concentration of 0.1%. This material was then applied to a 2.5 × 8 cm Cellex-D (Bio Rad) column. The column was then eluted with a 100 ml gradient of NaCl (0–0.75 M) in CMF-PBS containing 0.1% Triton X-100. Fractions containing activity were pooled and concentrated either by lyophilization or, if recovery of hemagglutination activity was desired, by dialysis overnight against Ficoll (Sigma). Samples were then boiled in sample buffer containing SDS and β-mercaptoethanol and electrophoresed in a 10% SDS polyacrylamide slab gel [10]. M_r standards included bovine serum albumin (M_r 68000), ovalbumin (M_r 43000), chymotrypsinogen (M_r 26000), and cytochrome *c* (M_r 13000). Gels were stained for protein with Coomassie blue. For the identification of ^{35}S-labeled proteins by fluorography, gels were stained as above, processed with Enhance (New England Nuclear), dried and then incubated at −70°C with Kodak X-Omat AR film. The film was developed with Kodak X-ray developer and fixative. Alternatively, gels were processed for recovery of hemagglutination activity according to Hager and Burgess [11] as described below.

2.3. *Localization of lectin activity in SDS gels*

Hemagglutination activity purified from conditioned medium by gel filtration and ion-exchange chromatography was concentrated by dialysis against Ficoll and then electrophoresed on SDS-polyacrylamide gels under reducing conditions as described above. Gels were sliced into 12 horizontal 0.5 cm strips, and each strip was cut into small pieces and placed into a 13 × 100 mm glass test tube. Protein was eluted from the fragments, precipitated in cold acetone, treated with 6 M guanidine HCl, and renatured according to Hager and Burgess [11]. After incubation for 3 h to allow renaturation of proteins, the 12 samples were assayed for hemagglutination activity. For M_r calibration, a vertical strip of the gel containing the protein standards was stained with Coomassie blue.

3. RESULTS

3.1. *Two-step purification of conditioned medium*

Hemagglutination activity was detected in conditioned medium prepared from teratocarcinoma stem cells and absent in control medium. This activity and that in the soluble cell extracts [5,6] were both inhibited by fucoidan and the mannan from the yeast mnn2 (0.03 and 3 μg/ml, respectively, for 50% inhibition), whereas other glycoconjugates, sulfated or not, including chondroitin sulfate, heparin, heparan sulfate, fetuin, and ovalbumin were ineffective at 100 μg/ml. These results suggest that the same lectin is present in the two sources. Because of the higher specific activity in conditioned medium (4-fold on a hemagglutination activity/mg protein basis), all subsequent studies were performed using this material. Hemagglutination activity of conditioned medium was excluded from Sepharose 2B (fig.1). SDS-PAGE of the excluded fraction and analysis by both fluorography and silver staining indicated that this material was enriched for a 56 -kDa polypeptide (not shown). Attempts to purify the lectin further were initially hindered by the rapid

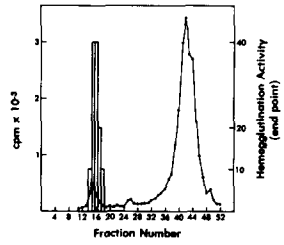

Fig.1. Sepharose 2B chromatography of conditioned medium. [^{35}S]Methionine-labelled conditioned medium was prepared as described in section 2 and 15 ml applied to the Sepharose 2B column. 50 μl samples of the fractions were counted (●), and hemagglutination activity determined (bars), as described in section 2.

loss of activity (50% after 1 day at 4°C) after gel filtration. Stabilization was achieved by adding Triton X-100 and maintaining the detergent at a final concentration of 0.1% through subsequent steps. When the material excluded from the 2B column (in 0.1% Triton) was applied to a Cellex-D (DEAE-cellulose) column, the hemagglutination activity was quantitatively adsorbed to the column and was eluted by 0.5 M NaCl (fig.2). The overall purification after this step was about 90-fold (table 1). Eluted fractions from the Cellex-D column were run on SDS-polyacrylamide gels and analyzed by fluorography. The pool containing the peak of hemagglutination activity contained as its major polypeptide a component with M_r 56000 (fig.3, lane C). This result was obtained in 4 independent experiments. No other components were consistently present. The 2 other protein peaks are not

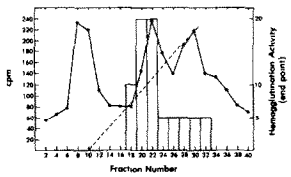

Fig.2. Cellex-D chromatography of the material excluded from the Sepharose 2B column. The fractions excluded from the 2B column containing the hemagglutination activity were pooled, Triton X-100 added, and the material applied to a Cellex-D column. The column was eluted with a linear salt gradient of 0–0.75 M NaCl from fraction 10 to 32 (– – –). 200 μl samples of the fractions were counted (●), and the hemagglutination activity determined (bars) as described in section 2.

Table 1
Hemagglutination activity in fractions purified from soluble extracts and conditioned medium

	Specific activity	% hemagglutination activity recovered	Purification factor
Conditioned medium (starting material)	0.015	100	–
2B-Void	0.06	25	4.0
Cellex-D, NaCl eluted	1.33	25	89.0

[^{35}S]Methionine-labeled conditioned medium was fractionated on Sepharose 2B and DEAE-cellulose (Cellex-D) as described in section 2. Specific activity is defined as hemagglutination activity per cpm in 10 μl of material

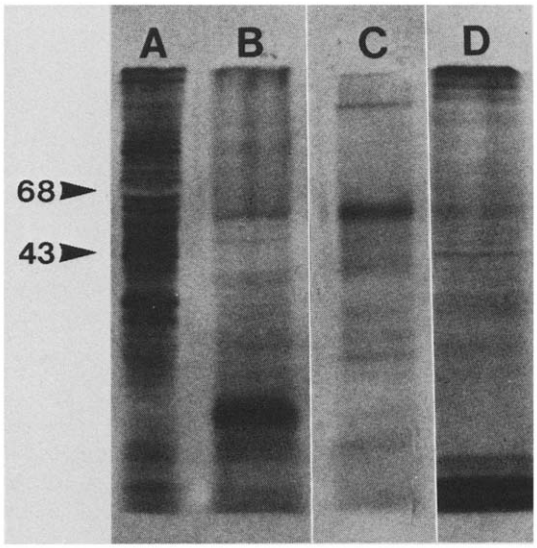

Fig.3. SDS-polyacrylamide gel of Cellex-D fractions. The fractions that constituted each of the 3 labelled protein peaks obtained after Cellex-D chromatography (see fig.2) were pooled and prepared for SDS-PAGE and fluorography as described in section 2. The gel was exposed for 3 weeks at −70°C. Lanes: (A) starting conditioned medium; (B) fractions 6–12; (C) fractions 19–25; (D) fractions 27–32. The numbers at the left indicate the molecular mass of the standards in kDa.

enriched for this band and contain little or no detectable hemagglutination activity (fig.3). The 56-kDa polypeptide was also the major band observed when gels containing this 2-step purified material were silver stained [12] for protein detection (not shown).

3.2. *Elution of hemagglutination activity from SDS-polyacrylamide gels*

To determine whether the 56-kDa polypeptide was responsible for the hemagglutination activity in the conditioned and fractionated medium, the purified material, after 20-fold concentration, was separated on a preparative SDS-polyacrylamide gel, the gel was sliced and the fragments eluted and renatured as described in section 2. In 4 independent experiments, we detected a single peak of hemagglutination activity that was localized in the 56-kDa region of the gel. Fig.4 represents one such experiment. The hemagglutination activity was generally not found in a single horizontal strip but in 2–3 strips. This spreading is probably due to the fact that protein bands on the preparative gel are

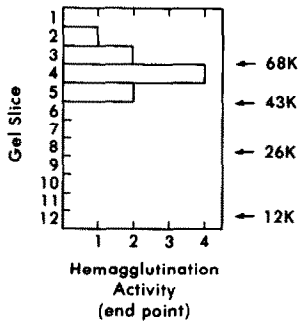

Fig.4. Recovery of hemagglutination activity after SDS-PAGE of 2-step purified conditioned medium. Two-step purified material obtained from conditioned medium was separated by SDS-PAGE and the gel was divided and assayed for hemagglutination activity (histogram) as described in section 2. The numbers at the right indicate the molecular mass of the standards (K, kDa) in a vertical strip of the gel (see section 2).

not parallel straight lines, but curve upwards at the edges of the gel. Also, gel slicing may have arbitrarily divided the activity into 2 sections. The yield of activity with this procedure ranged from 5 to 25% of the activity loaded onto the gel. The recovered hemagglutination activity was still inhibited by fucoidan, verifying that it represents the starting lectin activity. Based on these results we conclude that the lectin is composed of the 56-kDa polypeptide since there was no other labeled component consistently found within 12 kDa (1 gel strip) of this band (fig.3, lane C).

4. DISCUSSION

We report here the tentative identification of a 56-kDa polypeptide as the subunit of the teratocarcinoma stem cell lectin which we have previously described. The conclusion that the same lectin is responsible for the hemagglutination activity in soluble cell extracts and conditioned medium was supported by two observations. First, the hemagglutination activity in both exhibited the same pattern of inhibition by a panel of glycoconjugates. In addition, a 56-kDa polypeptide was the only component consistently enriched for when hemagglutination activity from either source was partially purified by gel filtration. The strongest evidence that this 56-kDa component was associated with the lectin is the observation that the hemagglutination activity in 90-fold purified conditioned medium could be consistently localized to the 56-kDa region following its elution and renaturation from a preparative SDS gel. The detection of the activity in a single region of the gel indicated that no additional subunits of different molecular masses are required for hemagglutination activity.

We plan to employ an antibody raised against the 56-kDa polypeptide eluted from SDS gels to substantiate our identification of this molecule as the lectin. This reagent will also allow immunocytochemical localization of the lectin during teratocarcinoma stem cell differentiation and mouse embryogenesis as well as further evaluation of the proposed role of the lectin in intercellular adhesion.

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