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Identification of a 25 kDa polypeptide associated with the L antigen in low potassium-type sheep red cells

Steven J. Pittman [1], J. Clive Ellory [1], Elizabeth M. Tucker [2] and Christopher I. Newbold [3]

[1] *University Laboratory of Physiology, Parks Road, Oxford,* [2] *Agricultural and Food Research Council, Institute of Animal Physiology and Genetics Research, Babraham, Cambridge and* [3] *Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford (U.K.)*

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Antisera to the L blood group antigen have been used, following radioiodination of low potassium-type sheep red cells and subsequent immunoprecipitation, to identify a polypeptide of the L antigen. Only LK, and not HK, cells express this 25 kDa component which is present in very low copy number.

The well-known genetic polymorphism of sheep red cells associated with intracellular potassium concentration (LK or HK type) has been shown to be linked to the ML blood group system, such that HK cells are homozygous MM and LK cells are homozygous LL or heterozygous ML [1,2]. The L antigen has been shown to inhibit the Na^+/K^+ pump in LK cells by altering the affinity for Na and K such that $K_{mK} < K_{mNa}$ [3]. The sheep alloantibody anti-L activates Na^+ pump fluxes in LK cells by altering these internal affinities back towards $K_{mK} > K_{mNa}$ [3]. Additionally, anti-L has effects on passive fluxes in LK cells, inhibiting the volume-sensitive KCl cotransport system [4]. This has led to the proposition that L activity can be separated into pump (L_p) and leak (L_l) components.

L has proved extremely refractory to purification. Although some preliminary detergent-solubilization data exist for the ML blood group system [5,6], it has been impossible to demonstrate L activity by immunoblotting extracts of LK red cells separated by SDS-PAGE, or by ELISA assays. This presumably results from a failure of the anti-L alloantisera to recognise epitopes of L other than in situ in the red cell membrane. There is also the problem of a low copy number for L (600 L_p and 850 L_l sites/cell) [7].

This paper presents evidence for the first time that L activity is associated with a 25 kDa polypeptide, using a sensitive ^{125}I cell-surface labelling technique, coupled with immunoprecipitation and autoradiographic detection.

Sheep red cells were washed three times in MBS (140 mM NaCl, 10 mM Mops (pH 7.4)) and the buffy coat removed. 100 μl of packed cells were surface-labelled with ^{125}I by a modification of the lactoperoxidase method [8]. Cells were resuspended to 1 ml in MBS with 100 nM KI. 0.16 units of lactoperoxidase were added, followed by 1 mCi of ^{125}I. The reaction was started by adding 12 μl of H_2O_2 (10 μl of 30% H_2O_2 in 10 ml MBS). 6 μl of H_2O_2 per minute were then added over 4 min. The reaction was stopped by washing the cells once in MBS with 50 μM sodium thiosulphate, followed by two washes in MBS with 10 mM KI. The cells were then washed extensively in MBS. Labelled red cells were then sensitized with excess anti-L or anti-M alloantisera (400 μl antiserum to 100 μl packed cells) by incubation at 32°C for one hour. Sensitized cells were then washed once in MBS and twice in NET (150 mM NaCl, 20 mM Tris-HCl (pH 7.4) with 5 mM EDTA). 100 μl cell pellets were extracted with 400 μl NETTI (NET buffer supplemented with 1% Triton X-100 and a cocktail of protease inhibitors; 1 mM PMSF, 0.01 mg/ml aprotinin, 0.01 mg/ml antipain, 0.01 mg/ml chymostatin, 1 mg/ml leupeptin, 0.2 mM TLCK and 0.1 mM TPCK) for 10 min at room temperature with stirring. Triton-insoluble material was removed by centrifugation at 13000 rpm for 5 min. 400 μl of Triton-soluble material was added to 100 μl of a 10% (v/v) suspension of protein-G Sepharose in NETT, and incubated overnight with shaking at 4°C. The protein-G Sepharose beads were then spun down gently at 1000 rpm for 10 s and washed

Correspondence: J.C. Ellory, University Laboratory of Physiology, Parks Road, Oxford, OX 3PT, U.K.

twice with NETT (with 1% (w/v) BSA), once in NETT, once in NETT (with 0.35 M NaCl), once in NETT, and twice in MBS. Gel samples were taken by boiling the protein-G Sepharose beads in Laemmli SDS-PAGE denaturation buffer [9]. Gel samples were run on 5–15 or 10–20% polyacrylamide linear gradient gels which were stained, destained, dried and subjected to autoradiography. Autoradiographs consistently revealed ^{125}I-labelled bands in all sensitised cells, both HK and LK (Figs. 1 and 2). Two of these were identified as erythrophilic IgG fragments (heavy and light chain corresponding to 50 kDa and 30 kDa) by immunoblotting using anti-sheep-IgG peroxidase conjugate with DAB visualization. The band at 48 kDa was variable, not always being present in LK cells and also appearing in some HK samples. Clearly this does not correlate with expression of the L antigen. The final band (of 25 kDa) was only found in LK sheep red cells sensitized with anti-L, HK cells always proving negative. Alloantisera raised against the sheep blood group antigen V also immunoprecipitate ^{125}I-labelled membrane components (of > 100 kDa) when incubated with serologically V-positive red cells (Fig. 2). V is thought to be a factor of the B blood group system in sheep and, although the function V is unknown, it is present in high copy number on the red cell surface and has been used as a positive control in the present study. Experiments using a panel of 4 different anti-L alloantisera, and a panel of 7 LK and 4 HK sheep, confirmed the identification of

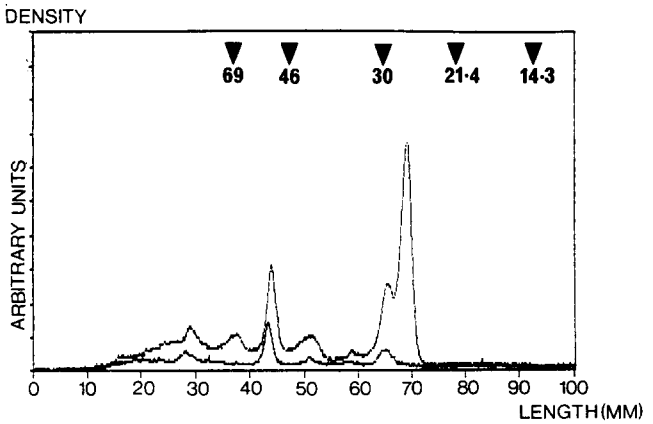

Fig. 1. Immunoprecipitation of L from detergent-extracted LK sheep erythrocytes. Superimposed densitometric scan of two tracks from an autoradiograph. The upper profile shows membrane components from Triton X-100-extracted LL erythrocytes, labelled with ^{125}I, sensitized with anti-L, and immunoprecipitated with protein-G Sepharose. The lower profile shows membrane components from Triton X-100-extracted MM erythrocytes, labelled with ^{125}I, sensitized with anti-L, and immunoprecipitated with protein-G Sepharose. Arrowheads indicate the position of molecular weight markers. Peaks at 50 kDa and 30 kDa show iodinated erythrophilic IgG heavy and light chain (identified by immunoblotting using anti-sheep IgG-peroxidase conjugate) present on the cell surface at the time of iodination. The major peak (at 25 kDa) represents the L antigen.

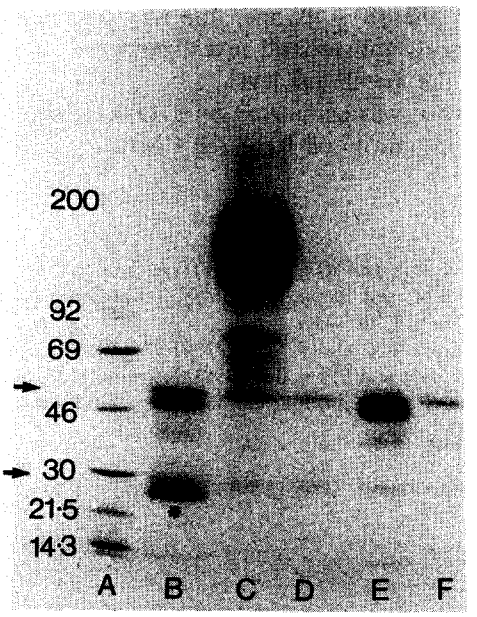

Fig. 2. Comparison between detergent-extracted LL and MM erythrocytes, labelled with ^{125}I, sensitized with antisera to L, M or V blood group antigens, and immunoprecipitated with protein-G Sepharose. Gel samples were separated by SDS-PAGE on a 5–15% linear gradient gel, which was stained, destained, dried and subjected to autoradiography using Amersham Hyperfilm-MP. (A) Molecular weight standards (Amersham ^{14}C-labelled Rainbow high molecular weight markers). Tracks B–F show immunoprecipitates. (B) Components of LL red cells sensitized with anti-L. (C) Components of LL, V-positive red cells sensitized with anti-V. (D) Components of LL red cells sensitized with anti-M. (E) Components of MM red cells sensitized with anti-L. (F) Components of MM, V-negative red cells sensitized with anti-V. Arrows show the position of erythrophilic IgG heavy and light chain. A polypeptide of 25 kDa (marked *) is recognised by antisera to the L antigen.

the 25 kDa polypeptide as associated with expression of the L antigen.

To attempt to answer the question whether this polypeptide is associated with L_p or L_l activity, we used an anti-L reagent known to have strong L_p but weak L_l activities (as defined by flux stimulation and serological assay, respectively) [10,11]. This produced significant labelling in the 25 kDa region, consistent with this band representing L_p activity.

The present finding of a 25 kDa polypeptide exclusively associated with the LK phenotype does not necessarily imply that this is the L antigen; it could represent a subunit or fragment of an oligomeric structure in the membrane. In fact, previous estimates of the size of L in the membrane by radiation inactivation gave a target size of 41 kDa [12], almost twice the present estimate. Human Rh(D) antigen has been shown to be 28.5 kDa [13]. These values fall in the size range 20–45 kDa corresponding to the Band 5–7 region by SDS-PAGE analysis.

Clearly these results open the way for characterization of the L antigen and investigation of its mode of action at the membrane level.

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