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TWO DETERGENT-INSOLUBLE PROTEINS OF THE HUMAN LYMPHOCYTE MEMBRANE ARE ENRICHED IN AN ISOLATED MEMBRANE FRACTION

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Human lymphocytes isolated from peripheral blood on Ficoll/Paque density gradients were surface-labelled by ^{125}I/lactoperoxidase or ^{3}H/reductive alkylation and lysed in buffer solutions containing non-ionic or amphoteric detergents (octylphenylpolyoxyethylenes, octylglucoside, cholylamidopropyldimethylammoniopropane sulfonate) under a variety of conditions. The cell lysate was fractionated by sedimentation or by density gradient centrifugation. The large majority of the labelled proteins is solubilized by the detergents. Two proteins of 45000 and 30000 molecular weight are the main detergent-insoluble, surface-labelled components. They can be fractionated from detergent lysates of cells in relatively pure form from the other membrane proteins and from nuclear material on density gradients. The same two proteins are specifically enriched in a membrane fraction isolated from a detergent-free cell homogenate by density gradient centrifugation. Cytoskeletal and other intracellular proteins remain associated with these two proteins when fractionated by either of these two independent methods.

Introduction

Non-ionic or amphoteric detergents, such as alkylphenylpolyoxyethylenes, octylglucoside or cholylamidopropyldimethylammoniopropane sulfonate, solubilize cell membranes by substituting the endogenous membrane lipids with a solvent medium for membrane proteins [1]. The cytoskeleton and the nuclei are the main components of the detergent-resistant fraction of cells lysed with non-ionic detergent [2]. Some membrane proteins, however, remain associated with the detergent-insoluble residue of cell lysates. This is generally assumed to be the result of an association of these proteins with the cytoskeleton [3,4]. This association may be transient and depend on a clustering of the membrane (receptor) proteins which may be induced by activating multivalent ligands or by enzymatic treatment [3,5,6,7].

In the present experiments two major proteins of the human lymphocyte membrane with molecular weights of 45000 and 30000 are characterized. They are specifically associated with the detergent-insoluble residue of the cell lysates and can be fractionated from nuclear material by density gradient centrifugation. Furthermore, surface membrane fractions can be isolated in the absence of detergents which are specifically enriched in these two proteins. Furthermore, it can be shown [8] that the 45 kDa protein is sensitive to neuraminidase treatment. A preliminary account of part of this work was presented elsewhere [9].

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Abbreviation: HLA, human lymphocyte antigen.

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Materials and Methods

Ficoll/Paque and Protein A from *Staphylococcus aureus* coupled to Sepharose CL-4B were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Nylon wool (LP-1 Leuko-Pak) was obtained from Fenwall Laboratories, Deerfield, IL., U.S.A. Lactoperoxidase (80 units/mg protein), glucose oxidase (type V), 1-*O*-*n*-octyl-β-D-glucopyranoside and cytochalasin b were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Ribonuclease (bovine pancreas, 100 units Kunitz/mg), desoxyribonuclease (bovine pancreas, 1000 units Kunitz/mg), phenylmethylsulfonylfluoride, Triton X-100/114 and 3-[(3-cholylamidopropyl)-dimethylammonio]-1-propane sulfonate were obtained from Serva Feinbiochemica, Heidelberg, F.R.G. NP-40 was obtained from BDH Chemicals, Poole, U.K. Carrierfree ^{125}I (100 mCi/ml) and NaB^3H_4 (5–15 Ci/mmol) were obtained either from the Radiochemical Center, Amersham, U.K., or from New England Nuclear, Boston, MA. U.S.A. En^{3}Hance was obtained from New England Nuclear, Boston, MA, U.S.A. An anti-β_2 microglobulin-antiserum (Nordic SwAHu/B2-mg) was obtained from Biogenzia Lemania, Lausanne, Switzerland.

Lymphocytes were isolated from buffy coats obtained from the Central Laboratory of the Swiss Red Cross in Bern. Buffy coats of usually 4–8 donors (blood groups O or A, Rh^+) were pooled at 4°C and processed within 1–2 h. They were suspended in phosphate-buffered saline (1.8 mM KH_2PO_4/8.2 mM $Na_2HPO_4 \cdot 2\,H_2O$/137 mM NaCl (pH 7.4)) containing 15% (v/v) 75 mM sodium citrate/38 mM citric acid/124 mM glucose, and sedimented (400 × *g*, 10 min, 20°C). The white cell enriched portion of the sediment was defibrinated by the addition of $CaCl_2$ (to 13 mM) and glass beads (gentle agitation, 10–15 min, 25°C). Lymphocytes were separated on Ficoll/Paque at 400 × *g* for 30 min at 20°C and suspended in phosphate-buffered saline containing 1.0 mg/ml glucose. The remaining erythrocytes were lysed with 0.83% NH_4Cl/10 mM $KHCO_3$/0.1 mM EDTA-Na_2 (10 min on ice). The lymphocytes were washed once more in glucose-containing phosphate-buffered saline. Generally, 97–98% of the cells were viable at this stage (Trypan blue exclusion). The cells were used either directly or purified by (a) incubation in minimum essential medium on a glass wool column to remove monocytic and dead cells ($2–3 \cdot 10^7$ cells per ml column volume for 30 min at ambient temperature or 37°C), or (b) incubation in minimum essential medium on a Nylon wool column mainly to remove B-lymphocytes (15 min at 37°C). After the glass wool column filtration, cell viability was > 99%. To exclude that the observed results were affected by the pooling of the buffy coats or by the defibrination, control experiments were carried out with cells of a single donor isolated without defibrination.

The cells were surface-labelled by lactoperoxidase-catalyzed iodination. 10 µl lactoperoxidase stock (1 mg/ml in phosphate-buffered saline), 10 µl glucose oxidase stock (4 units/ml in phosphate-buffered saline) and 100 µCi $N^{125}I$ were added per ml of cell suspension containing $2–5 \cdot 10^7$ cells/ml in glucose-containing phosphate-buffered saline. The labelling reaction was allowed to proceed for 30 min at ambient temperature. The cells were then washed (three times) with phosphate-buffered saline containing KI (to 5 mM). No decrease in cell viability was detected after the labelling reaction: for example cells filtered through glass wool prior to surface labelling remained > 99% viable.

In some experiments cells were surface-labelled by reductive alkylation. The cells were suspended in 200 mM sodium borate, 50 mM NaCl (pH 8.5) to give a final concentration of 10^8 cells/ml at 0–2°C. 100 µl of a freshly prepared 20 mM formaldehyde solution were added per ml cell suspension. After 30 s, 40 µl NaB^3H_4 (2 mCi) were added per ml cell suspension; the reaction was carried out at 0°C for 20 min. 100 µl of 10 mM $NaBH_4$ were then added per ml of cell suspension. After a further 20 min at 0°C the reaction was quenched by the addition of 5 ml ice-cold phosphate-buffered saline per ml of cell suspension. The cells were washed (twice) in the same buffer. Under these conditions no incorporation of ^3H into known intracellular proteins (e.g. actin) was detected. Protein standards were similarly labelled by reductive methylation [10].

Cells were lysed in a solution containing 150 mM NaCl, 50 mM Tris, 0.02% NaN_3 (w/v) [11]

and one of the following detergents: Triton X-100, NP-40 (0.5% w/v), Triton X-114 (1.0% w/v), octylglucopyranoside (30 mM) and cholylamidopropyldimethylammoniopropane sulfonate (10 mM) [12] at pH 7.4 on ice for 15 min. The cell lysates of 10^8 cells/ml lysis buffer were separated in a detergent-soluble supernatant and in a sediment containing nuclei and other detergent-insoluble material by centrifugation at $400–500 \times g$ for 10 min at 4°C.

The total detergent lysates of cells were fractionated on discontinuous sucrose density gradients as follows: successive steps of 60, 40 and 5% (w/v) sucrose in a solution containing 0.5% NP-40 or Triton X-100, 150 mM NaCl, 50 mM Tris and 0.02% NaN_3 at pH 7.4, were overlayered with a 0.5% Triton X-100 or NP-40 lysate and centrifuged at $97000 \times g_{av}$ for 2 h at 4°C (Sorvall AH 627 rotor). The nuclear pellet and the interphase fractions between 60/40% sucrose (T_3), 40/5% sucrose (T2) and from the top of 5% sucrose (T1) were recovered, washed in 5 volumes detergent buffer containing 0.1 mM phenylmethylsulfonylfluoride and pelleted at $260000 \times g_{av}$ for 1 h at 4°C (Sorvall T865 rotor).

Surface membrane fractions were isolated in the absence of detergents essentially according to the method of Monneron and D'Alayer [13]. Lymphocytes were suspended in a concentrated sucrose solution to obtain a final concentration of 60% sucrose determined by refractive index and homogenized in a glass-Teflon homogenizer with a tight fitting pestle at 3000–4000 rpm (8–10 strokes). The homogenate was adjusted to 40% sucrose, layered on a 73% sucrose cushion and overlayered, successively, with solutions containing 35%, 22.5% and 0% sucrose. All sucrose solutions contained 50 mM Tris, 25 mM KCl and 5 mM $MgCl_2$ (pH 7.4). The gradient was centrifuged in a Sorvall AH 627 rotor at $97000 \times g_{av}$ for 2 h at 4°C. Membrane fractions were recovered from the 22.5/0% sucrose (M1), 35/22.5% sucrose (M2) and 40/35% sucrose (M3) interphases. Each membrane fraction was diluted with 5 volumes of distilled water containing 0.1 mM PMSF and pelleted at $260000 \times g_{av}$ for 1 h at 4°C (Sorvall T865 rotor).

For gel electrophoresis urea (to 9 M) was added to the supernatant fraction of cell lysates. An equal volume of O'Farrell's buffer 'A' [14] (9.5 M urea/2.0% Triton X-100/2.0% ampholines (pH 3.5–10)/5.0% β-mercaptoethanol) was added before applying the sample to the gel. The detergent-insoluble fraction of 10^8 cells was incubated with 20 μl ribonuclease (5 units Kunitz/ml) and 20 μl desoxyribonuclease (1000 units Kunitz/ml) for 20 min at ambient temperature. Urea, detergent, ampholytes and β-mercaptoethanol were added as with the supernatant fraction. The remaining insoluble material was then removed by centrifugation before applying the sample to the gel. Membrane pellets and the T1-, T2-, T3-fractions were resuspended in O'Farrell's buffer 'A' [14].

The gel electrophoretic analysis was carried out in O'Farrell's two-dimensional gel system [14] with the following modification. If the cell lysate was obtained with a certain detergent X different from Triton X-100, the first dimension isoelectric focusing was carried out using a buffer system in which Triton X-100 was replaced by detergent X. ^{125}I-labelled gels were dried and exposed to Kodak X-Omat SO-282 X-ray film together with Dupont Cronex Li-Plus intensifier screens at −70°C. ^3H-labelled gels were processed with En^{3}Hance (NEN), dried and exposed to the same film at −70°C.

For immunoprecipitation of the histocompatibility antigens HLA-A,B 50 μl of undiluted anti-β_2-microglobulin were incubated with 200 μl of the detergent-soluble supernatant fraction of a cell lysate for 15 min on ice, added to 5 mg swollen Protein A coupled to Sepharose CL-4B in 100 μl phosphate-bufferd saline (containing 0.02% NaN_3/0.05 Triton X-100/1.0 $mg \cdot ml^{-1}$ bovine serum albumin) and incubated for a further 15 min on ice. The Sepharose was sedimented and washed 4 times with 1.0 ml phosphate-buffered saline containing 0.02% NaN_3, 0.05% Triton X-100, resuspended in O'Farrell's buffer 'A' [14] and applied to the gel.

The effect of cytochalasin b on membrane proteins was studied by suspending surface-iodinated lymphocytes at 10^7 cells/ml culture medium containing 5.0 μM cytochalasin b and incubating for 30 min at 37°C. The cells were then lysed in 0.5% NP-40 buffer and processed as above. In addition, the NP-40-insoluble sediment of control cells not

pretreated with cytochalasin b was incubated in an equivalent of 10^7 cells/10 μl phosphate-buffered saline containing 5.0 μM cytochalasin b.

Results and Discussion

Detergent lysates of cells

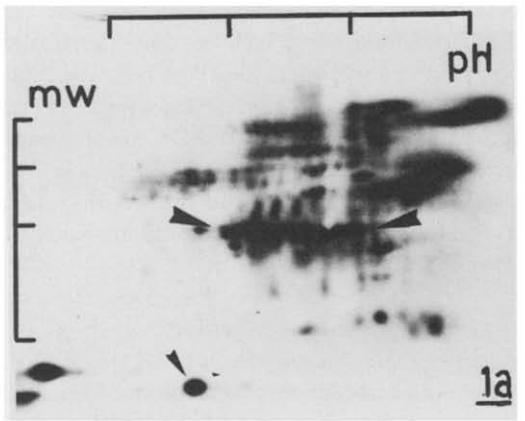
The gel pattern of the lymphocyte membrane proteins which are accessible to surface iodination are shown in Figs. 1 and 2. Fig. 1 refers to the soluble supernatant fraction of a 0.5% Triton X-100 cell lysate obtained after centrifugation at 400 × *g* for 10 min at 4°C. Among the many components, β_2-microglobulin and the histocompatibility antigens HLA-A and B were identified by immunoprecipitation with anti-β_2-microglobulin antiserum (Fig. 1c). Actin is the most prominent protein spot in the gel (Fig. 1b) from which the autoradiograph in Fig. 1a was taken. It can be recognized indirectly in the autoradiograph (Fig. 1a) by the slight displacement of the neighbouring histocompatibility antigens, HLA-A,B, but it is not surface-iodinated. It has been reported that actin in human B cells and, to a lesser extent, also in T cells may be exposed at the cell surface [15]. More recently, it has been reported that actin can be surface-labelled in stimulated, but not in resting normal T lymphocytes [16]. The data presented here confirm that actin in normal resting human T lymphocytes is not accessible to iodination by lactoperoxidase at the cell surface to a measurable extent.

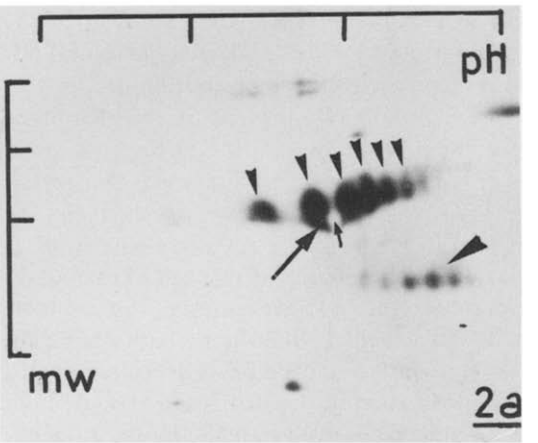
The detergent-insoluble 400 × *g* sediment of the cell lysate also contains surface-iodinated proteins (Fig. 2). Two proteins with apparent molecular weights of approx. 45 000 and 30 000 are prominent in this fraction. Both are heterogenic in charge. The main spots of the 45 kDA protein have isoelectric points from pH 4.5 to pH 5.7, but weaker spots are frequently seen both at more acidic and basic pH values. The isoelectric points of the 30 kDa protein are in the range from pH 4.2 to pH 5.0. Actin is the most prominent component also of the detergent-insoluble fraction (Fig. 2b). Although it again is not surface-labelled, it can be recognized indirectly in the autoradiograph by the slight displacement of the 45 kDa protein (Fig. 2a). In the region around actin, spots of slightly lower molecular weight are superimposed on the ovoid spots of the 45 kDA protein. From the location in the gel system, these spots would appear to be histocompatibility antigens, but not all of the isoelectric point subspecies of HLA-A and B are represented. Although HLA-A,B and the 45 kDa protein from the migration in the gel system thus appear to be separate molecules, the present data do not allow to exclude a relation of the 45 kDa protein (or of the 30 kDa protein) with gene products of the major histocompatibility complex. In addition, a few weaker spots in the higher molecular weight range are observed in Fig. 2a.

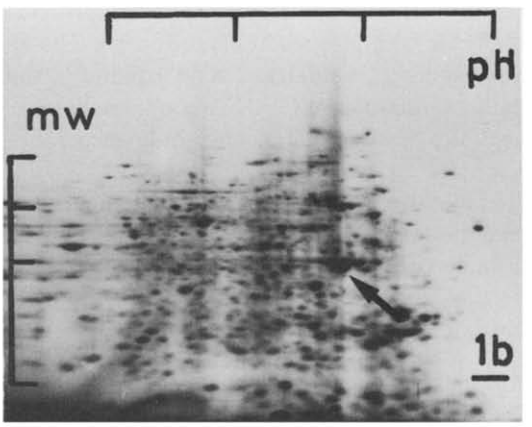
Analogous results were obtained with the pooled cells of several donors (see Methods) and with lymphocytes isolated from the blood of a single donor without defibrination, although blood platelets could never be removed entirely, when the defibrination step was omitted; the surface labelling pattern was then complicated by the platelet contamination of the cells. It has been reported that one of the several effects of cytochalasin b on human lymphocytes consists in the interference with the cytoskeletal function [17]. However, treatment of intact cells or of the insoluble sediment of lysed cells with cytochalasin b under conditions known to interfere with the cytoskeleton [17] had no effect on the exclusive association of the 45 kDA and 30 kDA proteins with the detergent-insoluble fraction.

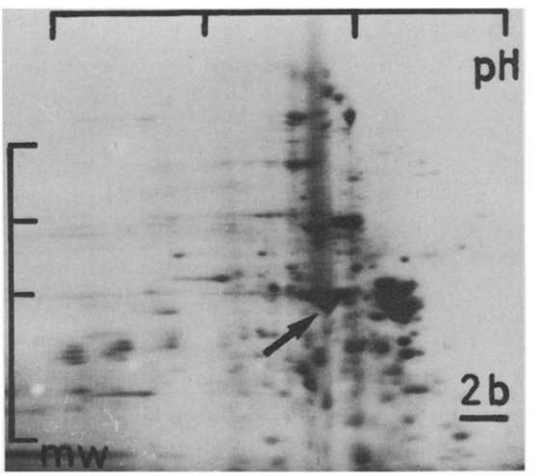
The 45 kDa and 30 kDa proteins partition within the limits of the sensitivity of the autoradiography exclusively into the insoluble fraction when solubilized by octylphenylpolyoxyethylenes. In parallel experiments with 0.5% or 1.0% Triton X-100 or X-114 lysates of the same number of cells, the 45 kDa and 30 kDa proteins are detected exclusively in the insoluble fraction. They are also largely insoluble in cholylamidopropyldimethylammoniopropane sulfonate and octylglucoside buffers, but traces of the 45 kDa protein are solubilized by these detergents.

The rapid rate at which the 45 kDA and 30 kDa proteins sediment in the detergent lysates indicates that these proteins may be either in the form of large protein aggregates or associated with cellular organelles. The analogy of the results obtained with three detergents of widely different chemical structure allow practically to exclude artefactual









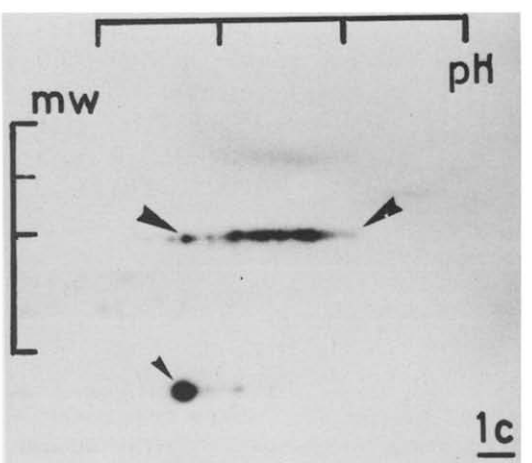


Fig. 2. Insoluble fraction of the 1.0% Triton X-114 lysate of surface-iodinated cells. (a) Autoradiograph of gel loaded with a sample equivalent to 10^8 cells. The 45 kDA protein (▾), the 30 kDa protein (▼), 'shadow' of actin (→), protein component overlapping with the 45 kDA protein (➡). (b) Parent gel stained for protein with Coomassie brilliant blue (actin, →). Molecular weight and pH scales same as in Fig. 1.

Fig. 1. 0.5% Triton X-100 soluble supernatant fraction of surface-iodinated cells. Histocompatibility antigens A and B (▼), β_2-microglobulin (▾), actin (➡). Molecular weight scale ($\times 10^{-3}$): 22, 45, 66, 116. pH: 4.0, 5.0, 6.0, 7.0 from right to left. (a) Autoradiograph of a gel loaded with a sample equivalent to $2 \cdot 10^7$ cells. (b) Parent gel stained for protein with Coomassie brilliant blue. (c) Immunoprecipitate with an anti-β_2 microglobulin antiserum and Protein A from lysed, surface-iodinated cells; autoradiograph of two-dimensional gel.

protein aggregations which are known to occur with some proteins in Triton X-100 buffers [18]. It is rather concluded that some specific property of the 45 kDa and 30 kDa proteins in the membrane renders them insoluble in the detergents used. Non-covalent intermolecular interactions appear to be involved, since the two proteins can be solubilized in 8 M urea or sodium dodecylsulfate (Fig. 2). In order to separate the 45 kDA and 30 kDA proteins from nuclear material, the complete lysate of cells labelled either by reductive methylation or by surface iodination, was layered on a discontinuous sucrose density gradient with steps of 60, 40 and 5% sucrose in 0.5% Triton X-100 or NP-40 buffer and centrifuged at $97000 \times g_{av}$ for 2 h at 4°C. A supernatant, the interphase fractions T1, T2, T3 and a nuclear pellet were recovered and analysed. It was found that the two main detergent-insoluble proteins are contained exclusively in fraction T2 in relatively pure form from other surface-labelled proteins (Fig. 3). In contrast to the 45 kDa protein, the 30 kDa protein was not labelled by reductive methylation of intact cells (Fig. 3). However, both the 45 kDa and 30 kDa proteins are detected in T2 isolated from surface-iodinated cells.

Other proteins, not surface-labelled and therefore presumably intracellular, are present in fraction T2. These can be detected when fraction T2, isolated from unlabelled cells is non-specifically tritiated by reductive methylation. From the relative spot intensities in autoradiographs (not shown) it can be estimated that the 45 kDa protein is of the order of a few percent of total protein in T2. None of the other fractions recovered from the density gradient contained detectable amounts of the 45 kDa and 30 kDa proteins.

Isolated surface membrane fractions

As reported by Monneron and D'Alayer [13], three main plasma membrane fractions M1, M2, M3 of different densities are obtained when detergent-free lymphocyte homogenates are fractionated by density gradient centrifugation. When analysed by gel electrophoresis and autoradiography, all three fractions contain surface-labelled proteins including the 45 kDa and 30 kDa proteins. The 45 kDa and 30 kDa proteins are mainly found in the two lightest fractions, M1/M2 (Fig. 4), but smaller amounts are also found in the third membrane fraction. However, the fractions M1/M2 are peculiar, because of all the surface-labelled proteins they contain almost exclusively the 45 kDa and 30 kDa proteins. (Fig. 4).

The autoradiograph in Fig. 4 was obtained from gels loaded with fractions M1/M2 isolated from

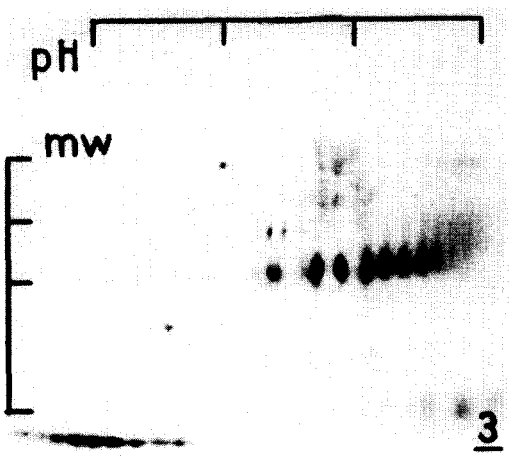


Fig. 3. Fraction T2. Cells surface-labelled by reductive methylation, lysed in 0.5% Triton X-100 and fractionated on density gradient in detergent. Autoradiograph of a gel loaded with sample equivalent to 10^9 cells. Molecular weight and pH scales same as in Fig. 1.

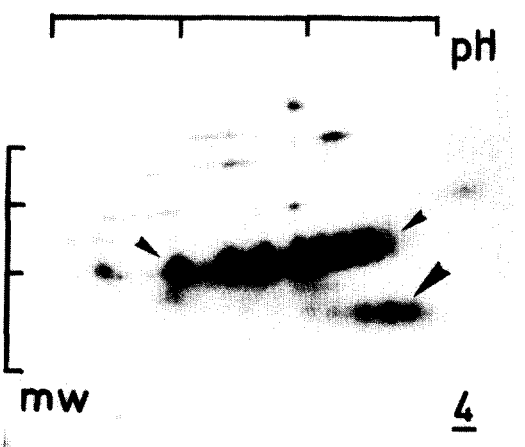


Fig. 4. Membrane fraction M1/M2 isolated from surface-iodinated cells. Autoradiograph. T = the 45 kDa protein (→), the 30 kDA protein (→). Molecular weight and pH scales same as in Fig. 1.

surface-iodinated cells. There is a large number of additional proteins present in fractions M1/M2, which can be visualized when M1/M2 isolated from unlabelled cells are non-specifically tritiated by reductive methylation (Fig. 5). Of these additional proteins, presumably intracellular, actin is the main component.

Isolated membrane pellets were solubilized in Triton X-100 buffers with detergent concentrations of up to 5 mg/mg total protein and sedimented (180000 × *g*, 1 h, 4°C). Gels and autoradiographs were obtained with the non-solubilized sediment which were practically identical with Fig. 2. It is concluded that the 45 kDa and 30 kDa proteins retain their detergent-insoluble property in the isolated membrane up to the high Triton X-100 ratio of 5 mg/mg protein. All essential components rendering the 45 kDa and 30 kDa proteins detergent-insoluble are contained in M1/M2; most likely, these components include actin and possibly other cytoskeletal elements [19] (Fig. 5), but none of the other major surface-labelled components with the possible exception of HLA-A,B.

The three membrane fractions isolated from surface-iodinated, homogenized cells on sucrose density gradients contained the following relative amounts of the total incorporated radioactivity:

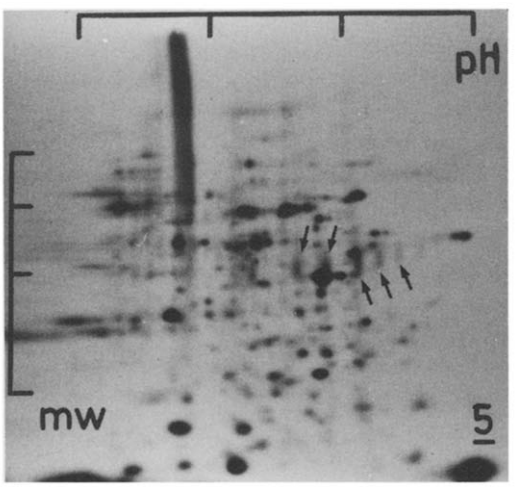


Fig. 5. Membrane fraction M1/M2 isolated from unlabelled cells. Reductive methylation of the membrane pellet after isolation. Autoradiograph. T = the 45 kDa protein (→). Molecular weight and pH scales same as in Fig. 1.

M1 2.3%, M2 5.3%, and M3 15.2%. These values are in good agreement with those reported by Monneron and D'Alayer [13]. Assuming homogeneous labelling probability of all membrane proteins, it can be estimated (Fig. 4) that the 45 kDa protein represents a few percent of total membrane protein.

Conclusions

There appears to exist a special class of membrane proteins defined by insolubility in non-ionic or amphoteric detergents [3–5]. The 45 kDa and 30 kDa proteins are the two main constituents of this class of proteins in T-cell enriched human lymphocytes (Fig. 2). They can be fractionated from solubilized and nuclear material by density gradient centrifugation of cell lysates, but remain associated with cytoskeletal and additional, intracellular proteins. The 45 kDa and 30 kDa proteins are specifically enriched in a cell membrane fraction isolated in the absence of detergents (Fig. 4), which also contains a number of unlabelled or intracellular proteins (Fig. 4). From preliminary electron microscopy studies, this fraction consists mainly of closed membrane vesicles. Some specific, if unknown property must be attributed to the 45 kDa and 30 kDa proteins to explain the observed detergent-insolubility. There is evidence from a number of studies (e.g. Refs. 2–6, 17) that a specific and functionally relevant but poorly understood property of cell membranes is reflected in the detergent-insolubility of some membrane proteins, which may be due to intermolecular interactions with cytoskeletal elements. It can be shown [8] that the 45 kDa protein is sensitive to neuraminidase treatment of cells or of isolated membranes and may partially dissociate the 45 kDa protein from the detergent resistant matrix, which may imply that the 45 kDa protein plays a role in the activation of mononuclear cells which can be induced by a treatment with neuraminidase and galactose oxidase [7].

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