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ISOLATION AND INITIAL CHARACTERIZATION OF A LYMPHOCYTE CAP STRUCTURE

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A method for isolating the cap structure induced by polycationized ferritin on the surface of mouse T-lymphoma cells is described. The procedure, based on the 'density perturbation' approach designed by Wallach and co-workers (Wallach, D.F.H., Kranz, B., Ferber, E. and Fischer, H. (1972) FEBS Lett. 21, 29–33), involves a simple, one-step density gradient centrifugation using metrizamide as the gradient material. The isolated polycationized ferritin cap fraction is approx. 20-fold enriched in plasma membrane relative to the whole cell homogenate and is apparently free of all uncapped membrane. Our initial analysis of the protein composition of the isolated cap structure indicates that there are approx. 30 membrane-bound polypeptides specifically associated with the polycationized ferritin cap fraction. Interestingly, there are at least four phosphorylated membrane-bound polypeptides (mol.wt. ~130 000, 100 000, 30 000 and 20 000) which are preferentially accumulated in the cap fraction. These findings provide further evidence for the selective redistribution of certain surface membrane proteins during lymphocyte capping.

Introduction

The binding of external ligands such as lectins, antibodies or cationized ferritin to the surface of lymphoid cells induces the spontaneous formation of local clusters or 'patches' of the ligand-receptor complexes. These patches can then form a large aggregate or 'cap' on the cell surface via an energy and temperature dependent process [1]. In recent year, this phenomenon of surface receptor redistribution has attracted at great deal of attention in the field of membrane biology [2]. Currently, the major research effort in this area appears to be focused on the association of cytoskeletal components (e.g. actin, myosin and tubulin) with surface receptor redistribution (so-called 'transmembrane interactions') [3], and on the aggregation of a variety membrane components during surface capping (so-called 'co-capping') [2].

Previously, surface receptor patching and capping have been described only at the morphological and immunocytochemical levels [4]. In order to better define the molecular structure of lymphocyte caps, we have recently developed a unique immuno-lactoperoxidase iodination procedure to specifically detect those proteins on the cell surface which are intimately involved in the cap structure [5]. However, since intracellular cytoskeletal components play an important role in receptor redistributions, it is necessary ultimately to isolate an intact structure in order to completely determine its molecular structure.

In this paper, we described a gentle and rapid method for cap isolation which utilizes the 'density perturbation' approach developed by Wallach and co-workers [6] with metrizamide as a density gradient material. In addition, we have begun to characterize the polypeptide composition of the isolated cap structure and have found that there is a preferrential accumulation of certain membrane-bound polypeptides in the cap fraction compared to the entire plasma membrane fraction. We belive that this is the first biochemical evidence for selective redistribution of certain membrane-assosiated components during lymphocyte capping.

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Materials and Methods

Cell culture. All experiments were carried out with a permanent mouse T-lymphoma cell line, designated R1G1 [7], which was obtained from Dr. R. Hyman, Salk Institute. Cells were cultured at 36°C in Dulbecco's Modified Eagle's medium supplemented with 10% heat-inactivated horse serum (GIBCO) and equilibrated in 5% CO_2/95% air. Under these conditions the cell culture maintain a generation time of about 18 h at densities between 10^4 and 10^6 cells/ml.

Induction of caps. Cell cultures (100 ml) were routinely harvested at a density of approx. $1 \cdot 10^6$ cells/ml and immediately chilled to 0°C. Cells were washed twice with 50 ml of ice cold, serum-free Dulbecco's Modified Eagle's medium and resuspended in 2 ml of cold Dulbecco's Modified Eagle's medium. Polycationized ferritin (Miles Lab.) was then added directly to the cell suspension with rapid mixing to obtain a final concentration of 0.5 mg/ml which is slightly above that required to saturate all the polycationized ferritin binding sites. Incubation of the polycationized ferritin-treated cell suspension for 90 min at room temperature (with occasional gentle mixing) induced capping of the bound polycationized ferritin on the surface of almost every cell (Fig. 1b) without any observable endocytosis occurring [8]. Over 90% of the capped cells were viable as determined by their ability to exclude trypan blue dye. Large aggregates, containing mostly dead cells, were removed from the sample by allowing them to settle out at 0°C for 5 min.

Isolation of caps. Capped cells were chilled to 0°C, washed twice with 10 ml of ice cold Dulbecco's phosphate buffered saline plus 5 mM $MgCl_2$ and resuspended in 2 ml of the same buffer. Mg^{2+} was included to stabilize nuclei against possible rupture during homogenization. Cell breakage was carried out at 0°C using a glass Dounce homogenizer fitted with the 'small' clearance pestle (Catalog number 357424, Wheaton Scientific). As monitored by phase contrast microscopy, more than 90% of the cells were broken after 75 strokes (done manually) without any significant numbers of nuclei being ruptured. A minor fraction of the nuclei appeared to have some plasma membrane left surrounding them.

The homogenate (generally 1 ml containing the equivalent of about $5 \cdot 10^7$ cells) was layered directly on top of a 4 ml discontinuous density gradient consisting of two 1 ml layers of metrizamide (Nygaard & Co.) dissolved in phosphate buffered saline (densities of 1.275 and 1.465 g/cm^3 at 5°C) overlayed with a 2 ml layer of 5% sucrose (w/v) in phosphate buffered saline. Metrizamide densities were calculated from their measured refractive indices based on the relationship: $\rho(5°C) = 3.453\ \eta(20°C) - 3.601$ determined by Rickwood and Birnie [9]. All gradients were centrifuged in a Backman SW 50.1 rotor at $100\,000 \times g_{av}$ for 90 min at 5°C and fractionated into three parts as follows: (1) a soluble fraction obtained by removal of the top 2 ml by pipet; (2) a particulate fraction banding at the interface of the sucrose and the lighter metrizamide band and (3) a cap fraction banding at the interface between the two metrizamide bands. The particulate and cap fractions were removed from the gradient by side-puncture using a 20 gauge needle and syringe to prevent any cross-contamination. No material sedimented through the dense metrizamide band.

Specific activity measurements. Because the majority of the protein in the cap fraction is due to polycationized ferritin (see Fig. 3), it was necessary to use a radioactive labeling procedure to determine the amount of cellular protein in that fraction. Cell cultures were incubated with 0.1 Ci/ml of a ^{14}C-labeled amino acid mixture (New England Nuclear, NEC-445) in the absence of polycationized ferritin for one to two generations before harvesting. All samples analyzed for ^{14}C content were extracted with hot trichloroacetic acid (90°C for 15 min), cold trichloroacetic acid (0°C for 5 min) and cold acetone (0°C for 5 min) in order to remove all radioactivity incorporated into non-protein components. The final precipitate was solubilized in 10% SDS (100°C for 10 min) and counted in 10 ml of Aquasol II (New England Nuclear) plus a total of 1 ml aqueous sample. We determined that cellular proteins were uniformly labeled to about the same extent by first directly measuring the total protein content in the homogenate and soluble fractions using the Coomassie Blue procedure of Bradford [10], and then calculating the amount of ^{14}C/mg of protein in both fractions. Since this number was almost identical for both fractions in any particular experiment, we conclude that all the cellular proteins are uniformly labeled to

about the same extent and, therefore, one is justified in using the ^{14}C content as a relative measure of the amount of cellular protein in any fraction.

^{125}I-labeled samples were counted directly in a gamma spectrometer and quench corrections due to the presence of high concentrations of metrizamide were made when necessary. (Na^+, K^+)-ATPase activity was assayed using freshly prepared material and the procedures of Wallach and Kamat [11] and Ames [12].

Plasma membrane isolation. Plasma membrane material was isolated by the method described by Johnson and Bourne [13]. Cells were harvested by low speed centrifugation ($500 \times g_{av}$), washed twice with Dulbecco's phosphate buffered medium and resuspended at a density of 10^7 cells/ml in 150 mM NaCl, 20 mM Hepes, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM 2-mercaptoethanol, pH 7.4 at 4°C. Cells were disrupted by Dounce homogenizer as described above. The lysate was centrifuged at $750 \times g_{av}$ for 5 min and the supernatant was centrifuged in a Beckman SW 50.1 rotor at $43\,000 \times g_{av}$ for 20 min. Pellet was resuspended in 10% sucrose (w/w), 20 mM Na-Hepes, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM 2-mercaptoethanol, pH 8.0 and layered over a discontinuous gradient of 20%, 30%, 40% and 50% sucrose (w/w) in the same buffer and centrifuged in a Backman SW 50.1 rotor at $100\,000 \times g_{av}$. The membrane between 30% and 40% was collected, and washed with Dulbecco's phosphate buffered saline.

Phosphorylation. Cells ($1 \cdot 10^6$ cells/ml) were first washed with phosphate free Dulbecco's modified Eagle medium. Subsequently, 0.2 mCi ^{32}P ($H_3\,^{32}PO_4$, carrier free, from ICN) was added to cell suspension at room temperature for 30 min. Phosphorylation was terminated by adding an excess amount (10 vol.) of unlabeled 0.1 M phosphate-saline buffer at 0°C. Phosphorylated cells were then induced to form polycationized ferritin-mediated cap structure. Phosphorylated plasma membrane and cap fractions were isolated according to the procedures described above.

SDS-polyacrylamide gel electrophoresis and radioactivity analysis. Electrophoresis was carried out with slab gels using the discontinuous buffer system described by Laemmli [14] and an exponantial polyacrylamide gel gradient (7.0–17.5%). All samples were dissolved in a buffer containing 2% SDS, 0.1 M dithiothreitol, 0.003% bromophenol blue, 20 mM Tris-HCl, pH 8.9 and heated at 100°C for 2 min Approx. 125 μg protein per sample (e.g., total plasma membrane or cap fraction) was applied to the gel. Electrophoresis was run at a constant current of 2 mA for 18 h at room temperature and the polypeptide banding pattern revealed by staining with Coomassie Blue in the case of samples labeled with ^{32}P the gels were dried and exposed to Kodak X-ray film.

Immunofluorescence microscopy. For visualizing polycationized ferritin binding sites at the light microscopic level, polycationized ferritin-treated cells were first fixed with 2% paraformaldehyde in phosphate-saline buffer for 30 min at 0°C, washed with phosphate-saline-glycine, and labeled with rabbit antibodies against ferritin and fluorescein-conjugated goat antibodies against rabbit immunoglobulin as described previously [8]. To illustrate the initial distribution of surface polycationized ferritin binding sites in prefixed samples, cell suspension was fixed with 2% paraformaldehyde, followed by the treatment with polycationized ferritin and immunoreagents for localizing ferritin as described above.

Electron microsocpy. Polycationized ferritin-treated cells and isolated cap structures were fixed with 2% glutaraldehyde, and then post-fixed with 1% osmium tetroxide (both buffered with 0.1 M sodium cacodylate (pH 7.0)) each for 1 h at 4°C. Cell pellets were then dehydrated through a graded ethanol series and embedded in Epon 812. Ultrathin sections were examined without any further staining with uranyl acetate or lead citrate using a Philips 301 electron microscope operating at 80 kV.

Results

Formation of polycationized ferritin caps

Initially, we established that the distribution of polycationized ferritin on prefixed cells was rather uniform over the entire cell surface (Fig. 1a). Incubation of unfixed R1G1 cells with polycationized ferritin for 90 min at room temperature induces the formation of polycationized ferritin-cap structures in more than 90% of the cell population (Fig. 1b). This result was confirmed by indirect immunofluorescence staining of the polycationized ferritin-treated cells with rabbit antibody against ferritin followed by fluorescent-labeled goat antibody against rabbit immunoglobulin (Fig. 1a and 1b). Polycationized fer-

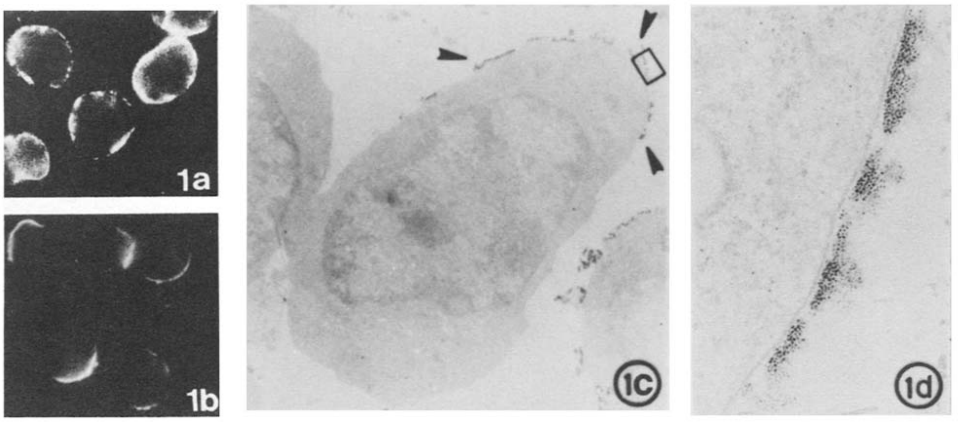


Fig. 1. Polycationized ferritin labeling on the surface of mouse T-lymphoma cells. Indirect immunofluorescence staining of uncapped (a) and capped cells (b) using rabbit anti-ferritin followed by fluorescein-labeled goat anti-rabbit IgG. Magnification: × 1 300 for (a) and (b). Electron micrographs of capped cells (c) and (d). Staining with uranyl acetate was omitted in order to allow clear visualization of the polycationized ferritin. Magnification: × 6 000 and × 100 000 for (c) and (d), respectively. Details of the procedures for both types of microscopy are given in Ref. 8.

ritin-capped cells were then examined in more detail using the transmission electron microscope. A typical capped cell is shown in Fig. 1c. In particular, it should be noted that large clusters of electron-dense polycationized ferritin are aggregated over the surface of one region of the cell. The section delineated by the small rectangle in Fig. 1c is shown at higher magnification in Fig. 1d. It is clear that the clusters of polycationized ferritin within the capped region are separated by gaps containing very little, if any, polycationized ferritin. The large, multilayered clusters of polycationized ferritin are apparently formed during the capping process because unbound polycationized ferritin is not aggregated and pre-fixed R1G1 cells bind polycationized ferritin rather uniformly in a more or less continuous monolayer (data not shown). It is not known yet how these large clusters of polycationized ferritin are formed. Other investigators have also observed similar types of polycationized ferritin clusters [15–19].

Isolation of the polycationized ferritin cap fraction

We have utilized the heavy density and capping ability of polycationized ferritin together with the 'density perturbation' approach of Wallach et al. [6] to develop a simple and rapid method for the isolation of a lymphocyte cap structure. Briefly, the procedure involves gentle lysis of the capped cells in an isotonic buffer using a Dounce homogenizer followed by centrifugation of the whole cell homogenate on a metrizamide discontinuous density gradient.

Cell breakage was carried out under isotonic conditions in order to prevent the denaturation or loss of possibly labile membrane-associate structures which might occur under the hypotonic conditions generally used with Dounce homogenization. It is likely that capped cells are readily broken in an isotonic buffer because their outer membrane is more fragile than that of uncapped cells. Metrizamide was chosen for the density gradient material because it has the following important advantages over the more common density gradient materials such as sucrose, Ficoll and CsCl: (1) higher densities can be obtained without extremely high viscosity and (2) its non-ionic nature with lower osmolarity at high densities reduces the possibility of denaturing labile biological material [9].

In order to determine the density at which the cap structure band in metrizamide, caps were formed using polycationized ferritin pre-labeled with ^{125}I via a standard lactoperoxidase labeling procedure [20]. First, iodinated polycationized ferritin was shown to induce the normal capping reaction on the surface of the T-lymphoma cells. Then, we determined that the density of the polycationized ferritin cap structures ranged from approx. 1.260–1.330

TABLE I

DISTRIBUTION OF CAPPED ^{125}I-LABELED POLYCATIONIZED FERRITIN

Capped cells were formed using polycationized ferritin prelabeled with ^{125}I via lactoperoxidase labeling technique [20]. Cells were homogenized and overlayed with 2 ml layer of 5% sucrose on a discontinuous metrizamide density gradient (1.275 and 1.465 g/cm^3 at 5°C). After the centrifugation at 100 000 × g_{av} for 90 min in a Beckman SW 50.1 rotor at 5°C, gradients were fractionated into (1) a soluble fraction obtained by removal of the top 2 ml of sucrose solution (2) a particulate fraction banding at the interface of the sucrose and the lighter metrizamide band (1.275 g/cm^3) and (3) a cap fraction banding at the interface between the two metrizamide bands (1.275 and 1.465 g/cm^3). All three fractions as well as an aliquot of homogenate were counted directly in a gamma spectrometer and quench corrections due to the presence of high concentrations of metrizamide were made when necessary.

Fraction	^{125}I radioactivity	
	Total ^{125}I (cpm)	%
Homogenate	2920	100
Soluble	110	3.8
Particulate	1240	42.5
Cap	1680	57.5

g/cm^3 at 4°C in metrizamide (data not shown). However, it was also found, using cells which were not treated with polycationized ferritin, that some contaminating cellular material bands at densities up to about 1.275 g/cm^3 in metrizamide. Consequently, we have selected the cap fraction as the material banding at densities greater than 1.275 g/cm^3. Typically, about 60% of the polycationized ferritin cap structures is recovered in this fraction (Table I). It is possible that some of the cap structures with densities less that 1.275 g/cm^3 originate from the edge of the cap and thereby have a lower ratio of polycationized ferritin: membrane than those structures originating in the central region of the cap. It is also possible that some of the polycationized ferritin cap structures banding at the sucrose/metrizamide interface are just physically trapped at that position by the accumulation of particulate cell components.

In order to assess the purity of the isolated cap fraction, capping was induced with polycationized ferritin (unlabeled) on cells whose surface proteins were previously iodinated with ^{125}I. The results of a typical cap isolation are presented in Table II. Approx. 55% of the total ^{125}I-labeled proteins is recovered in the cap fraction. Using iodinated cells not treated with polycationized ferritin, only about 3% of the total counts loaded on the gradient is recovered at the metrizamide interface where the cap fraction bands. In addition, the following experiment was performed in order to determine whether some of the radioactively labeled material present in the cap fraction could have been artifactually trapped with or bound to the dense polycationized ferritin cap structures during the isolation procedure. Unlabeled, polycationized ferritin-capped cells were mixed with an equal number of ^{125}I-labeled, uncapped cells; the mixture of cells was homogenized and run on the standard metrizamide gradient to obtain the cap fraction. As shown in Table II, the dis-

TABLE II

DISTRIBUTION OF ^{125}I-LABELED CELL SURFACE PROTEINS

In order to assess the purity of the isolated cap fraction, certain experiments were carried out as follows: (A) capping was induced with polycationized ferritin on cells whose surface proteins were previously iodinated with ^{125}I; (B) ^{125}I-labeled cells were not treated with polycationized ferritin; (C) non-radioactive, polycationized ferritin-capped cells were mixed with an equal number of ^{125}I-labeled, uncapped cells. All samples were homogenized and processed through the same procedures as described in Table I to obtain the distribution of ^{125}I-labeled materials in homogenate, soluble, particulate and cap fractions.

Fraction	Radioactivity (%) in experiment		
	A + polycationized ferritin	B – polycationized ferritin	C + polycationized ferritin, unlabeled plus – polycationized ferritin, labeled
Homogenate	100	100	100
Soluble	4.2	4.5	4.1
Particulate	41.9	91.9	93.4
Cap	53.4	3.4	2.5

TABLE III

RELATIVE SPECIFIC ACTIVITIES OF ^{125}I-LABELED SURFACE PROTEINS AND (Na^+,K^+)-ATPase

In order to find out the extent of enrichment of plasma membrane in cap structures, two plasma membrane markers such as ^{125}I-labeled surface proteins and (Na^+,K^+)-ATPase were used for the test. ^{125}I-labeled surface proteins were carried out by lactoperoxidase technique [20]. ^{14}C-labeled amino acid mixture was added to cell cultures for one to two generations to label all the cellular proteins. (Na^+,K^+)-ATPase activity was assayed using the procedures of Wallach and Kamat [11] and Ames [12]. The relative specific activities for ^{125}I-labeled proteins or (Na^+,K^+)-ATPase were calculated as the ratio between ^{125}I and ^{14}C or the ratio between units and ^{14}C, respectively. The numbers in parenthesis are the factor by which the specific activities increase in the 'cap' fraction relative to the homogenate.

	Homogenate	Cap
^{125}I (cpm/ml)	$5.1 \cdot 10^5$	$3.0 \cdot 10^5$
^{14}C (cpm/ml)	$6.3 \cdot 10^3$	$1.87 \cdot 10^2$
Rel. spec. act. (^{125}I/^{14}C)	81	1600 (19.8x)
(Na^+,K^+)-ATPase (units/ml)	4.0	6.7
^{14}C (cpm/ml)	$3.70 \cdot 10^3$	$1.09 \cdot 10^2$
Rel. spec. act (units/^{14}C)	$1.1 \cdot 10^{-3}$	$6.16 \cdot 10^{-2}$ (56x)

tribution of labeled surface proteins from the uncapped cells is unaffected by the presence of the capped cell homogenate. If there were artifactual binding or trapping occurring, one would expect to observe an increase in the amount of labeled membrane recovered in the cap fraction. Therefore, these data strongly support the conclusion that uncapped surface membrane is not collected in the cap fraction to any significant extent.

We have also confirmed that the isolated cap fraction is highly enriched in plasma membrane by determining the specific activities of the ^{125}I-labeled surface proteins (i.e. ^{125}I cpm/mg cellular protein) and of the (Na^+, K^+)-ATPase (a specific marker enzyme for plasma membrane [21]) in the cap fraction. As shown in Table III, both of these specific activities are significantly greater in the cap fraction compared to the total homogenate.

Electron microscopic analysis of the polycationized ferritin cap fraction

Examination of the isolated cap fraction in the electron microscope (Fig. 2a–c) reveals membrane-bound polycationized ferritin clusters separated by gaps which appear almost identical to those observed on the surface of whole capped cells (Fig. 1c and d). Except for a very small number of polycationized ferritin-free membrane vesicles, all of the structures visualized in the cap fraction are membranes coated with clusters of polycationized ferritin. In addition, all of the observed closed membrane vesicles have polycationized ferritin bound on the outside indicating that inside-out vesicles are apparently not formed during cell breakage. Upon thorough examination of a large number of sections cut through the 'cap' fraction, no structures were observed which resembled either whole cells, nuclei, mitochondria, Golgi, rough endoplasmic reticulum or ribosomes. The only non-membraneous material observed in the cap fraction is some fibrous-like structures attached to the inside of the polycationized ferritin-vesicles (see the small arrowheads in Fig. 2a and b). We conclude from these images that the material visualized in the cap fraction originated almost exclusively from the polycationized ferritin-capped region of the cell. We believe this method can generally be applied to the isolation of any type of cap. For example, ferritin can be conjugated to almost any particular ligand and the dense cap structure isolated in the same manner described above.

Biochemical analysis of the polycationized ferritin cap fraction

As a first step in characterizing the proteins present in the polycationized ferritin cap structure, both the total plasma membrane fraction and the cap fraction were analyzed by SDS-polyacrylamide gel electrophoresis. All samples were run on 7.5–15% polyacrylamide gradient slab gels [14] and the protein bands were detected by Coomassie Blue staining and/or autoradiography. As shown in Fig. 3, the cap fraction (Fig. 3B) contains a large number of polypeptides (approx. 30 protein bands) but their banding pattern clearly differs from that of the total plasma membrane fraction (Fig. 3A).

Since it is already known that phosphorylated molecules play important regulatory roles in mem-

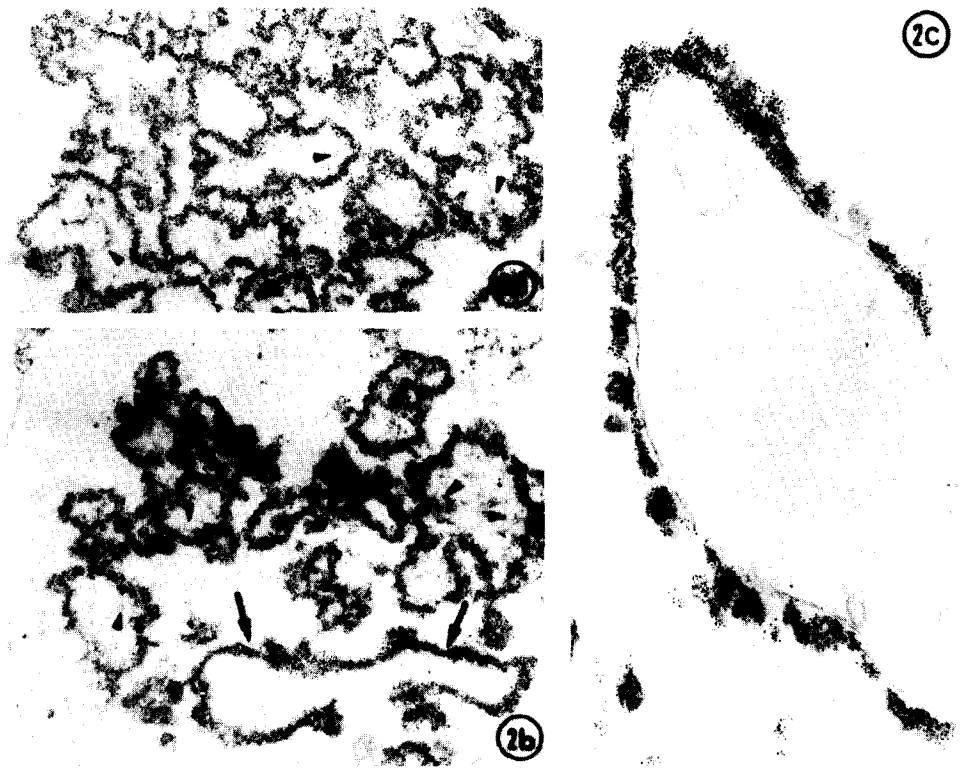
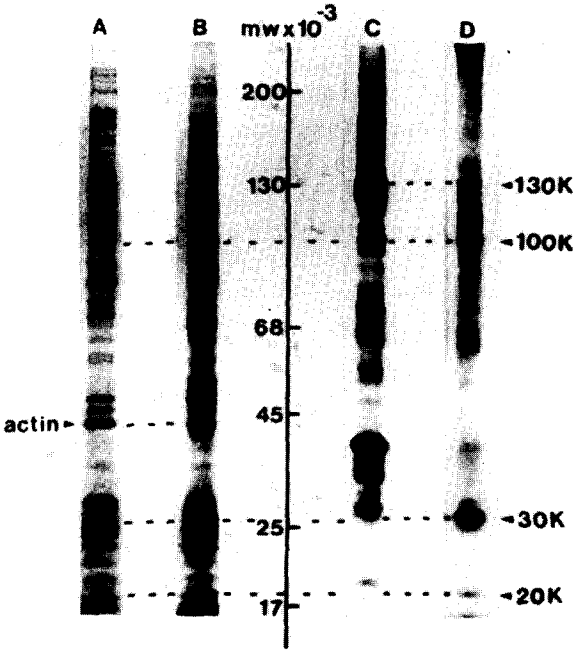


Fig. 2. Electron micrographs of the 'cap' fraction. Large arrows in (b) indicate an open membrane sheet in contrast to the more prevalent closed membrane vesicles. Small arrowheads in (a) and (b) indicate fibrous-like material apparently attached to the inner surface of the membrane vesicles. Magnification: X 30 000 for (a) and (b) and X 100 000 for (c).



brane-cytoskeletal interactions [22–25], it was obviously of interest to determine whether phosphorylated polypeptides are present in the cap structure. By labeling of the cell with ^{32}P followed by SDS-polyacrylamide gel electrophoresis plus autoradiographic analysis of the isolated cap structures and entire plasma membrane, we have found that approx. 20 membrane-bound polypeptides in the total plasma membrane fraction are phosphorylated (Fig. 3C). However, only four of these phosphorylated poly-

Fig. 3. SDS-polyacrylamide gel and autoradiographic analysis of plasma membrane and 'cap' fraction. The plasma membrane is shown in A and C and the 'cap' fraction in B and D. A and B are stained with Coomassie Blue; C and D are autoradiograms of the phosphorylated proteins. Molecular weight marjer proteins (myosin, 200 000; β-galactosidase, 130 000; bovine serum albumin, 68 000; actin, 45 000; concanavalin A, 25 000; and soybean inhibitor, 17 000) were run in parallel channels on the same gel.

peptides (mol.wt. ~ 130 000, 100 000, 30 000 and 20 000) are preferrentially accumulated in the polycationized ferritin-cap structure (Fig. 3D). Therefore, it is clear from these data that selective redistribution of some surface membrane proteins must occur during the capping induced by the rather non-specific polycationized ferritin ligand.

Discussion

Until the present time, the phenomenon of lymphocyte capping has been primarily analyzed by a variety of cytochemical methods [2]. Most of the conventional ligands (e.g., antibodies against specific receptors and lectins) are known to induce only a fraction of the total cell population (at most 30–50%) to form cap structures rather asynchronously [2]. Consequently, it is very difficult to obtain precise biochemical information concerning the cap structures. In order to define the molecular organization of a cap structure, one needs to employ a ligand which induces a very high percentage of the cell population to form caps at a relatively synchronous rate.

Recently, we have reported that polycationized ferritin (which has been used in the past as an electron-dense marker for determining surface anionic sites) behaves like most conventional multivalent ligands with respect to its ability to induce receptor redistribution on the surface of lymphoid cells [8]. This polycationized ferritin-induced capping is both energy- and temperature-dependent in addition to being closely associated with actin-containing cytoskeletal components [8]. Most importantly, polycationized ferritin is able to induce almost 90% of the cell population to form cap structures synchronously within a short incubation time (10–15 min at room temperature) [8]. Therefore, we believe that the synchronous and quantitative nature of polycationized ferritin-induced capping provides an excellent model system for biochemical studies on the cellular components involved in lymphocyte surface cap information.

In this paper we have presented a simple and rapid procedure for the isolation of the cap structure induced on the surface of mouse T-lymphoma cells treated with polycationized ferritin. Using only a one-step density gradient centrifugration in metrizamide, one can obtain a cap fraction which is almost totally free of all other cell components. It contains approx. 60% of the polycationized ferritin originally bound to the surface of the cells (Table I) and is enriched about 20-fold in plasma membrane compared to the whole cell homogenate (Table III).

By labelling the surface proteins (primarily tyrosin-containing molecules) with ^{125}I before incubation with polycationized ferritin, we have determined that almost 55% of the total surface label is recovered in the cap fraction (Table II). Since recovery of the cap structure itself is estimated to be about 60%, one must conclude that approx. 90% of the labeled surface protein (i.e. 55/60) is collected into the cap. At first glance, this seems to be a very large fraction of the total surface protein. However, it is not so unreasonable in view of the fact that polycationized ferritin binds a large percentage of the surface proteins due to their predominantly negative charge.

Previously, a transmembrane interaction mechanism has been proposed to explain how all surface receptor capping occurs [3]. Specifically this mechanism involves both membrane-associated actin and myosin which are directly or indirectly bound to an integral protein or class of proteins in the plasma membrane (called X protein(s)). Upon the addition of external ligands (e.g. antibodies against specific receptors of lectins), membrane receptors are aggregated and become bound to X protein(s). The receptor aggregates through X protein(s), linked to actin and myosin, are then collected into a cap by a sliding filament mechanism analogous to muscle contraction [3]. So far, very little evidence for or against such a transmembrane interaction mechanism has been obtained. Using mouse lymphocytes, Flanagan and Koch [26] have recently shown that capping of Ig receptors induces a specific association between surface Ig receptors and intracellular actin. Condeelis [27] has also reported that a concanavalin A cap structure isolated from the slime mold, *Dictyostelium discoideum,* contains a large amount of actin and myosin. However, detergents were used in the isolation procedures in both of these experiments, and so it is quite possible that some of the membrane components were lost during the cap preparation. To our knowledge, this paper is the first report of a cap structure being isolated without the use of detergents. Obviously, this was done in an attempt to determine

the full complement of cap components, including both membrane and cytoskeletal elements.

Analysis of the transmembrane interactions occurring between surface receptor molecules and cytoskeletal components requires knowledge of the protein molecules oriented towards the cytoplasmic side of the membrane. The technique which we have employed for labeling such proteins is simply to allow the cells to incorporate inorganic $^{32}PO_4$ so that labeled phosphorylation (in vivo) takes place only on the cytoplasmic side of the plasma membrane. Two examples of this technique are: (1) transmembrane proteins, HLA-A, HLA-B, and T-200 are readily phosphorylated at cytoplasmic side of the plasma membrane [28,29]; and (2) cytoskeletal components, spectrin and myosin which are known to be closely associated with plasma membrane are also phosphorylated at the cytoplasmic side of the membrane [22,23,25]. In this study, the internal ^{32}P-labeling studies indicate that approx. 20 polypeptides in the total plasma membrane fraction are phosphorylated from cytoplasmic side of the membrane. Most interestingly, only four of these phosphorylated polypeptides (e.g., 130-K, 100-K, 30-K and 20-K protein) are preferrentially accumulated in the cap structure. Although we have not yet firmly identified any of these phosphorylated bands present in the cap fraction (Fig. 3D), preliminary data indicate that the two larger molecular weight proteins, 130-K and 100-K, are either integral or transmembrane proteins (to be published elsewhere). In addition, the 30-K and 20-K proteins appear to correspond to the two myosin light chain molecules as characterized by the myosin antibody-mediated immunoprecipitation technique described previously [25]. Furthermore, we have recently observed cytochemically that actin is accumulated directly underneath polycationized ferritin caps on the surface of the T-lymphoma cells [8]. Our present experiments also indicate that actin may be present in the isolated polycationized ferritin cap fraction (Figs. 3A and 3B). These data with regard to the co-accumulation of both integral/transmembrane proteins and actin/myosin in the cap structure all support the existence of 'transmembrane interactions' which have been proposed to play crucial roles in the capping mechanism [3].

Using an immunobiochemical approach we have recently determined that both myosin light chains (30-K and 20-K protein) are extensively phosphorylated and preferentially attached to the plasma membrane of capped cells [25]. The fact that the 30-K and 20-K polypeptides (myosin light chain proteins) are also present in the cap structure suggests that phosphorylation of the light chain myosin may also induce the actin-activated Mg^{2+}-ATPase of myosin light chains to collect receptors into cap formation (analogous to the actin-myosin sliding filament mechanism in muscle cells). However, it is not yet clear whether either the 130-K, the 100-K protein, or both are part of the so-called 'X protein(s)'which have been proposed to be either directly or indirectly associated with actin and myosin [3]. It is also possible that other important cap-associated components (both membrane proteins and cytoskeletal elements) are not detected by the phosphorylation technique we have employed. Further analysis and characterization of the cap-associated polypeptides and their relationships with actin/myosin are currently underway.

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