Ligand-independent cap formation: Redistribution of surface receptors on mouse lymphocytes and thymocytes in hypertonic medium*

(immunoglobulin/H-2 antigens/concanavalin A receptors/scanning electron microscope/microvillus)

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ABSTRACT Most of the mobile receptors on mouse lymphocytes and thymocytes, including immunoglobulins, H-2 antigens, Thy-1.2 antigens, some concanavalin A receptors, and some antigenic determinants detected by anti-thymocyte serum, were redistributed into caps when the cells were incubated in hypertonic medium (about 800 mOsm) in the absence of ligands. The cap formation could be induced with the addition of ligands if the cells were defrayed again to isotonic medium. The viability of the cells was not decreased after incubation in the hypertonic medium. Ligand-independent cap formation appeared to depend upon cellular metabolism. Different species of receptors appeared to move with different mobilities during the process of ligand-independent cap formation. Most microvilli on cells showing caps in hypertonic medium were associated with the regions of the caps. These results suggest that free receptors can be induced to form caps if the receptors are allowed to interact with the machinery of cap formation under special conditions.

Cell surface receptors are distributed randomly in the plane of the membrane but can be redistributed into patches and caps after crosslinking by multivalent ligands (1). Segregation of crosslinked receptors from other receptors is a characteristic of ligand-dependent cap formation. For instance, when surface immunoglobulins (Ig) on mouse lymphocytes are crosslinked with antibody directed against immunoglobulin (anti-Ig) and then capped, other surface molecules (e.g., alloantigens such as H-2 antigens) still remain distributed randomly on the cell surface (2).

In contrast to the common cap formation described above, exceptional cases have been reported to be independent of ligands, including cap formation of viral antigens of tumor cells (3, 4) and that observed on lymphocytes stimulated with lipopolysaccharide (5). The former, however, occurs only after prolonged incubation (up to 4 hr) in tissue culture medium (3) and the latter has been observed mainly on stimulated B-lymphocytes that were undergoing movement (5). One therefore might be tempted to conclude that ligand-independent cap formation is not a basic property of biological membranes.

We report here the finding that cap formation of most of the movable surface receptors on lymphocytes can take place in hypertonic medium without addition of ligands. Ligand-independent cap formation appeared to be induced on a major population of these cells soon after their transfer into hypertonic medium. We also found that different species of receptors moved with different mobilities in the process of ligand-independent cap formation. In addition, studies by both transmission and scanning electron microscopy of the morphology of those cells showing caps in hypertonic medium indicated a strong association of the caps with microvilli. These results are discussed in terms of both receptor–cytoplasmic interactions and of the mechanism of cap formation.

MATERIALS AND METHODS

Preparation of Cell Suspension. Phosphate-buffered saline (PBS) has been described (6). In the present study, we used 2 × PBS as a hypertonic medium; this is composed of 2-fold amounts of each component of PBS.

ICR mice, which were randomly bred within a closed colony in Shizuoka Laboratory Animal Center (Shizuoka, Japan), were generally used as sources of splenic lymphocytes. Thymocytes and splenic lymphocytes prepared from BALB/c mice (provided by T. Matsuhashi, University of Tokyo) were used if necessary. Cells were washed with PBS and suspended in the indicated media. Viability of cells determined by trypan blue exclusion was around 90% even after exposure to 2 × PBS at 37° for 15 min.

Fluorescein and Ferritin-Labeled Antibodies. Anti-mouse IgG (anti-Ig) was produced in rabbits and conjugated with fluorescein isothiocyanate as described (6). Ferritin-labeled anti-Ig was prepared and purified according to the method described by de Petris and Raff (7). Anti-H-2d sera (C57BL/10 anti-B10.D2 and (C57BL/10 × AKR.M)F1 anti-B10.A) were kindly provided by G. M. Edelman and R. Milner (The Rockefeller University). Fractions precipitated from the H-2 sera at 40% saturation of ammonium sulfate were conjugated with fluorescein isothiocyanate. Thy-1.2 antigens on thymocytes were detected by indirect immunofluorescence, using anti-Thy-1.2 serum (AKR anti-C3H; Searle Diagnostic, Buckinghamshire, England) and fluorescein-labeled rabbit Ig directed against mouse IgG (fl-anti-Ig).

Ligand-Independent Cap Formation in 2 × PBS. Cells were incubated in 2 × PBS at 37° for 10 min and fixed in 1% formaldehyde in 2 × PBS at 25° for 10 min. They were then washed in 2 × PBS and incubated with 100 μg ml−1 of fl-anti-Ig in 2 × PBS at 25° for 10 min. Redistribution of Ig was observed as will be seen below. Redistribution of Ig was not observed (i) if cells were fixed in 1% formaldehyde in PBS at 25° for 10 min, incubated at 2 × PBS at 37° for 10 min, and labeled with fl-anti-Ig, or (ii) if cells were incubated in 2 × PBS containing 20 mM NaNO2 fixed in 1% formaldehyde in 2 × PBS containing NaNO2, and labeled with fl-anti-Ig in 2 × PBS. Fixation of cells in 1% formaldehyde appeared, therefore, to be sufficient to inhibit redistribution of Ig under the conditions used in the present study. Labeling of fixed cells with fluorescent antibodies

Abbreviations: PBS, phosphate-buffered saline; Ig, immunoglobulin; fl-anti-Ig, fluorescein-labeled rabbit Ig directed against mouse IgG; Con A, concanavalin A.

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was specific, because fluorescein-labeled normal rabbit IgG did not bind to fixed lymphocytes that had been incubated in 2 X PBS.

**Fluorescence Microscopy.** Fluorescence microscopic observations were carried out with an Olympus fluorescence microscope model BH-RFL with an HBO 100W/2 (Osram) light source. To determine the percentage of stained cells showing caps, at least 200 stained cells were observed. Photographs were taken with Kodak Tri-X film with an exposure time of 30 sec.

**Transmission Electron Microscopy.** Cells to be examined were fixed with 2% glutaraldehyde in PBS (or in 2 X PBS, if necessary) at 25°C for 1 hr and postfixed in 1% osmium tetroxide in 0.1 M collidine buffer (pH 7.2) at 25°C for 1 hr. The cells were dehydrated and embedded in Epon 812 mixtures. The sections of cells were examined with a JOEL JEM-100C electron microscope.

**Scanning Electron Microscopy.** Cells to be examined were fixed with 2% glutaraldehyde in PBS (or in 2 X PBS, if necessary) at 25°C for 1 hr, washed with PBS, and adsorbed onto cover slips coated with polylysine (8). The cells were dehydrated in ethanol, washed in amyl acetate, and dried by the critical point method with CO2 as the transition fluid using a Hitachi Critical Point Dryer HCP-1. The specimens were coated with a thin layer (200–250 Å) of gold in an Eiko Ion Coater 1B-3 and observed with a Hitachi S500 scanning electron microscope operated at 25 kV with 100-sec scanning periods. Photographs were taken with Fuji Neopan SS-12 films.

**RESULTS**

**Immunoglobulin Cap Formation in Hypertonic Medium in the Presence of Anti-Ig.** When mouse splenic lymphocytes were incubated at 37°C with 100 μg ml⁻¹ of fl-anti-Ig in 2 X PBS, the morphology of caps was quite different from that of caps formed in isotonic medium (PBS) under the same conditions. Most of the former capped cells appeared to have one small, dot-like, bright fluorescent area per cell (Fig. 1b), whereas the latter exhibited a crescent-shaped distribution of fluorescence (Fig. 1d). The cap region of cells capped in hypertonic medium was observed in the transmission electron microscope to be extraordinarily villous (Fig. 2a). When ferritin-labeled anti-Ig was used, ferritin molecules appeared to be packed in the spaces between microvilli (Fig. 2b).

**Immunoglobulin Cap Formation in Hypertonic Medium in the Absence of Anti-Ig.** In the course of kinetic studies on cap formation in hypertonic medium, we found that cap formation of surface Ig had taken place before addition of anti-Ig. Mouse lymphocytes were incubated at 37°C for 10 min in 2 X PBS and fixed with formaldehyde. The fixed cells were washed and labeled with fl-anti-Ig. Up to 70% (but generally around 50%) of the labeled cells showed caps (Table 1).

Incubation of lymphocytes in PBS containing 0.2–0.4 M sucrose without addition of fl-anti-Ig also resulted in cap formation of surface Ig. The frequency of ligand-independent cap formation in those media containing sucrose, however, was relatively lower than that obtained in 2 X PBS (Table 1), probably because the inhibitory effect of high concentrations of sugars on the mobility of cell surface proteins (9) might have partially canceled the ligand-independent redistribution of Ig in the medium with high osmolarity. About 500–600 mOsM medium was found to induce optimal ligand-independent cap formation (Table 1).

A subpopulation of lymphocytes that were incubated in 2 X PBS without addition of anti-Ig showed only a part of the sur-
Table 1. Ligand-independent cap formation* under various conditions

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Medium</th>
<th>Cap, %†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Splenic lymphocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ig</td>
<td>PBS</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1.5 × PBS</td>
<td>10 ± 1</td>
</tr>
<tr>
<td></td>
<td>2 × PBS</td>
<td>56 ± 12</td>
</tr>
<tr>
<td></td>
<td>2.5 × PBS</td>
<td>3 ± 1</td>
</tr>
<tr>
<td></td>
<td>2 × PBS + 20 mM NaN₃</td>
<td>1 ± 1</td>
</tr>
<tr>
<td></td>
<td>2 × PBS + 10 μg ml⁻¹ of cytochalasin B</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2 × PBS + 100 μg ml⁻¹ of Con A²</td>
<td>2 ± 2</td>
</tr>
<tr>
<td></td>
<td>2 × PBS + 0.1 mM colchicine + 100 μg ml⁻¹ of Con A</td>
<td>10 ± 3</td>
</tr>
<tr>
<td></td>
<td>2 × PBS → PBS²</td>
<td>3 ± 3</td>
</tr>
<tr>
<td></td>
<td>PBS + 0.1 M sucrose</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>PBS + 0.2 M sucrose</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>PBS + 0.3 M sucrose</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>PBS + 0.4 M sucrose</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>PBS + 0.5 M sucrose</td>
<td>0</td>
</tr>
<tr>
<td>H-2</td>
<td>2 × PBS</td>
<td>38</td>
</tr>
<tr>
<td>Con A</td>
<td>2 × PBS</td>
<td>9</td>
</tr>
<tr>
<td>receptors</td>
<td>2 × PBS + 0.1 mM colchicine</td>
<td>16</td>
</tr>
<tr>
<td>ATSI binding</td>
<td>2 × PBS</td>
<td>3</td>
</tr>
<tr>
<td>sites</td>
<td>2 × PBS + 0.1 mM colchicine</td>
<td>9</td>
</tr>
<tr>
<td>Thymocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-2</td>
<td>2 × PBS</td>
<td>44</td>
</tr>
<tr>
<td>Thy-1.2</td>
<td>2 × PBS</td>
<td>36</td>
</tr>
</tbody>
</table>

* Cells were incubated in indicated media at 37° for 10 min, fixed, and labeled with 100 μg ml⁻¹ of fl-anti-Ig.
† Cap formation is expressed as percent of the total stained cells. In some cases, mean ± SEM is given.
‡ Cells were incubated with concanavalin A (Con A) in PBS and transferred to 2 × PBS containing Con A.
§ Cells were incubated with colchicine at 37° for 30 min in PBS.
* Cells were incubated in 2 × PBS at 37° for 10 min and incubated in PBS at 37° for 10 min.
² Anti-mouse thymocyte serum.

face Ig redistributed into caps; the residual part of the Ig remained distributed diffusely over the cell surface (Fig. 3).

Requirements for Ligand-Independent Cap Formation in Hypertonic Medium. Ligand-independent cap formation in 2 × PBS was strongly inhibited by 20 mM NaN₃, 10 μg ml⁻¹ of cytochalasin B, or 100 μg ml⁻¹ of concanavalin A (Con A) (Table 1). Colchicine (0.1 mM) partially reversed the inhibitory effect of Con A on ligand-independent cap formation, suggesting that colchicine-sensitive structures are involved in the inhibition of receptor mobility by Con A in the present system as well as in anti-Ig-induced patch and cap formation (10).

Reversion of Ligand-Independent Cap Formation by Lowering the Osmolarity of Medium. Lymphocytes incubated at 37° in 2 × PBS were transferred to PBS and incubated at 37° for different periods. The cells were fixed with 1% formaldehyde in PBS and labeled with fl-anti-Ig in order to examine the distribution of surface Ig. Whereas 56% of the stained cells with fl-anti-Ig showed caps if the cells were fixed before transfer of the cells to PBS, only 3% showed caps when fixed after incubation for 10 min in PBS (Table 1). This suggests that Ig caps formed independently of ligand in hypertonic medium can revert to a diffuse distribution in isotonic medium. In addition, we have observed intermediate distributions from cap to diffuse such as those shown in Fig. 3 among the cells transferred from 2 × PBS to PBS. Neither 20 mM NaN₃ nor 10 μg ml⁻¹ of cytochalasin B inhibited the reversion from the capped to the diffuse state. This observation suggests that the reversion from capped to diffuse state might be accounted for by diffusion of molecules in the plane of membrane. If cells were incubated in 2 × PBS in the presence of fl-anti-Ig, the formed caps were not reversed after transfer to PBS.

Ligand-Independent Cap Formation by Other Receptors. We have found that H-2 antigens on splenic lymphocytes and thymocytes and Thy-1.2 antigens on thymocytes were capped in 2 × PBS without addition of any antibody (Table 1). Parts of the populations of both Con A receptors and antigenic sites for anti-mouse thymocyte sera on each cell also appeared to be capped by 2 × PBS. Treatment of lymphocytes with 0.1 mM colchicine increased the proportions of those receptors capped in 2 × PBS on each cell as well as the number of cells showing distinct ligand-independent cap formation of those receptors. The details of the results will be published elsewhere.

Simultaneous Movements of Cell Surface Receptors and Microvilli in Ligand-Independent Cap Formation. One of the features of cap formation in hypertonic medium is the accumulation of microvilli in the cap region. Cells with randomly distributed microvilli were common among populations that had not been exposed to hypertonic medium. In contrast, most of the microvilli in cells in hypertonic medium appeared to be localized (Fig. 4) in regions that coincided with caps, as observed in transmission electron microscopic studies with ferritin-labeled anti-Ig. This cell morphology was rarely observed when cap formation was induced by ligands in PBS. On some cells capped in hypertonic medium, a small number of microvilli could be seen in regions other than the cap.

Transfer of cells from 2 × PBS to PBS decreased the number of cells with highly localized microvilli and, instead, gave populations of cells showing nonuniform but not highly localized distributions of microvilli (Fig. 5). Cells showing this type
of distribution of microvilli probably correspond to those showing the intermediate distribution of Ig described above.

**Patch Formation in Hypertonic Medium.** Within the resolution of fluorescence microscopy, apparent patch formation was not detected on cells that were incubated in 2 × PBS containing NaN₃, fixed, and labeled with fl-anti-Ig. In order to examine the possibility that patch formation might be nonspecifically induced in hypertonic medium without addition of ligand but not detected in the microscope, we studied specific patch formation of receptors by ligands in 2 × PBS. When lymphocytes were incubated in 2 × PBS containing NaN₃ and further incubated with 100 μg ml⁻¹ of fl-anti-Ig in the same medium, patch formation of Ig was detected in the fluorescence microscope. This result suggests that spontaneous patch formation by Ig receptors does not occur in hypertonic medium.

**DISCUSSION**

The present report describes cap formation that can be induced on a large fraction of lymphoid cells by simple incubation in 2 × PBS. This phenomenon, which occurs independently of crosslinking ligands, has been observed for Ig, H-2 antigens, Con A receptors, and anti-mouse thymocyte serum reacting sites of mouse lymphocytes and for H-2 and Thy-1.2 antigens of thymocytes. Media made hypertonic with sucrose also induced cap formation without addition of ligands, suggesting that the high osmolarity of medium is the cause of the ligand-independent cap formation.

The differential mobilities of various receptors observed in 2 × PBS (Table 1) can be explained in terms of the hypothetical receptor–cytoplasmic interactions originally proposed by Edelman *et al.* (11, 12). According to this hypothesis, the mobility of surface receptors is negatively controlled by interaction with cytoplasmic structures, which are assumed to consist of microtubules, microfilaments, and other structures. The stimulatory effect of colchicine on Con A-independent cap formation of Con A receptors (Table 1) supports this view.

As shown in Fig. 3, not all of the surface Ig molecules were capped in some populations of cells. This fact suggests that a portion of Ig molecules might be interacting with the cytoplasmic structures described above. This interaction would appear to be disrupted when Ig molecules are crosslinked by anti-Ig, in that all of the Ig on a single cell was capped in the presence of anti-Ig. Alternatively, some Ig molecules might have failed to interact with a contractile system that positively controls the mobility of receptors and directs the process of cap formation. In this case, a portion of the Ig would remain diffusely distributed while at the same time those Ig molecules that did interact with the contractile machinery would be capped.

Association of caps with microvilli has been clearly demonstrated for cap formation in 2 × PBS irrespective of the presence of anti-Ig. Although the casual relationship between the presence of microvilli and the mobility of receptors is not clear, it is very likely that a unique mechanism directs the movements of both receptors and microvilli. The microvilli appeared to move with the receptors even when cellular metabolism was suppressed, for metabolic inhibitors such as NaN₃ did not prevent the disappearance in isotonic medium of ligand-independent caps or microvilli that had accumulated in the cap region. The mechanism of movement (or disappearance and reappearance at the different place) of microvilli remains puzzling.

Since Taylor *et al.* (1) found that cap formation can be induced by specific ligands, crosslinking of receptors by multi-
valent ligands (patch formation) has been thought to be a prerequisite to cap formation. Hypotheses (13, 14) for the mechanism of cap formation were therefore required to explain how the crosslinked receptors moved independently of free receptors. Although we have clearly demonstrated that ligands are not necessary to induce cap formation of receptors under the conditions described here, our results are not sufficiently detailed to rule out the possibility that receptors might be clustered in hypertonic medium and, therefore, be induced to form caps. After incubation without anti-Ig in 2 × PBS or the same medium containing NaN₃, no patch formation of Ig was detected in the present study within the resolution of fluorescence microscopy. In addition, preliminary electron microscopic studies with ferritin-labeled anti-Ig suggest that Ig molecules are randomly distributed on cells that had been fixed after incubation in 2 × PBS containing NaN₃. However, the density of Ig was low near the tops of microvilli (unpublished observations). These results do not exclude the possibility that patch formation was induced in hypertonic medium, because such patches should include a variety of different receptors and, therefore, each patch would contain only a small number of Ig molecules. Nevertheless, the absence of ligand-independent patch formation in hypertonic medium is suggested by the fact that specific patch formation of Ig directed by fl-anti-Ig can be detected in 2 × PBS containing NaN₃. Furthermore, this conclusion is supported by the observation that receptors accumulated in the region of caps in the absence of ligands were quickly dissociated, probably by diffusion, when capped cells were transferred from hypertonic medium to isotonic medium. From these observations, it can be tentatively concluded that patch formation of receptors is not a prerequisite to cap formation in hypertonic medium.

Ligand-independent cap formation was inhibited by NaN₃, cytochalasin B, or Con A, all of which are inhibitors of ligand-dependent cap formation (2). Therefore, the cellular machinery that directs ligand-dependent cap formation, which appears to depend on crosslinking of receptors, also appears to work in the process of ligand-independent cap formation observed in hypertonic medium.

The question therefore arises as to why patch formation appears to be a prerequisite to cap formation in isotonic medium but not in hypertonic medium. We can speculate that in isotonic medium patches but not free receptors are able to interact with the machinery of cap formation, whereas both patches and free receptors can interact with this machinery in hypertonic medium. We observed that the cell volume was decreased and the average number of microvilli per cell was increased when cells were transferred from isotonic medium to hypertonic medium (unpublished observations). It seems possible therefore that the membrane structures, including circumferential or cortical cytoplasmic structures, might be perturbed in hypertonic medium so that even free receptors can interact with the cytoplasmic contractile structures that comprise the machinery of cap formation. The possibility that membrane structures are also perturbed in hypertonic medium remains to be proven.

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