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BIOCHEMICAL ANALYSIS OF LIGAND-INDUCED RECEPTOR PATCHING AND CAPPING USING A NOVEL IMMUNOLACTOPEROXIDASE IODINATION TECHNIQUE

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ABSTRACT

A novel approach for the analysis of membrane proteins involved in ligand-induced surface receptor patching and capping is described. The technique is based on the use of immunolactoperoxidase (immuno-LPO) conjugates which catalyze the iodination of those surface proteins with available tyrosine groups that are located in the immediate vicinity of the patch or cap of a particular antigen. We have used the patching and capping of the *H-2* (histocompatibility) antigen on mouse thymocytes to illustrate this method. However, this technique should be generally applicable to any cell surface proteins which can be induced to form patches or caps by a specific ligand.

Cytochemical analysis indicates that the immuno-LPO conjugates induce the same patching and capping of the *H-2* antigen as does the unconjugated antibody. Biochemical analysis of the ^{125}I-labeled proteins by SDS polyacrylamide gel electrophoresis indicates that a large membrane protein (mol wt of ~200,000 daltons) is closely associated with *H-2* patches and caps. Since a number of other prominent membrane proteins are not labeled by this procedure, selective redistribution of certain surface proteins must be occurring during *H-2* antibody-induced patching and capping.

KEY WORDS   immunolactoperoxidase iodination · patching/capping · surface *H-2* antigen redistribution · immunofluorescence · thymocyte · SDS polyacrylamide electrophoresis · EM autoradiography

The animal cell membrane is obviously the site of a number of important immunological and communicative reactions. Among them are the phenomena of ligand-induced patching and capping of receptors on the surface of mammalian fibroblasts and lymphoid cells. One major question in this area of membrane biology is whether the redistribution of a particular surface receptor involves other specific proteins on the plasma membrane. With respect to lymphocytes, the conventional approach to this problem is to induce the capping of a first component and then cytochemically locate a second component relative to the first. If the two components are not associated on the membrane, the patching and capping of one may not alter the distribution of the second component. On the other hand, if both components are physically linked or become associated during patching or capping, then both should redistribute together, i.e., co-cap.

It has been reported by some laboratories that capping of lymphocyte surface immunoglobulin (Ig) leads to the co-capping of the F_c receptor (1, 5). However, surface Ig molecules appear to redis-

tribute independently of the C_3 receptor, major histocompatibility (MHC) (HLA in man, or H-2 in mouse) antigens and I_a antigen (2, 3, 13, 14, 19, 23, 24). In addition, similar types of experiments have shown that mouse H-2^K, H-2^D, and I_a antigens all redistribute independently of one another (11, 23, 24). On the other hand, we have recently shown, using a double immunofluorescence technique, that H-2 and related antigen molecules appear to co-cap with a variety of different cell surface antigens such as viral glycoprotein gp69/71 and T200 antigen (a major glycoprotein in thymoma cells and T-lymphocytes) (4). These investigations concerning the interrelationships between surface molecules during their redistribution have been restricted to a two-molecule situation, as studied by immunofluorescence techniques at the light microscope level. Very little biochemical information has been obtained concerning other membrane components possibly involved in receptor patches and caps. Therefore, our understanding concerning the mechanism of patching and capping is still at a preliminary, descriptive stage.

The primary aim of this paper is to describe a new approach using immunolactoperoxidase (immuno-LPO) conjugates for identifying those surface proteins which are directly involved in membrane receptor patching and capping. We have used the specific case of the mouse thymocyte-H-2 antigen to illustrate this method. The H-2 antigens on the surface of mouse lymphocytes are known to be involved in various immune reactions, particularly T-lymphocyte responses (7). Consequently, it is of considerable interest to explore the nature and topographical arrangement of any membrane components possibly involved in the redistribution of surface H-2 molecules induced by specific antibodies. However, this method should be generally applicable to any cell surface receptor that can be induced to form patches and caps by a specific ligand.

METHODS AND RESULTS

H-2 Antigen Patching and Capping

Mouse thymocytes used in all the experiments described in this paper were obtained from the thymus of C57BL/6J strain mice. All cells were washed with RPMI 1640 medium (Grand Island Biological Co., Grand Island, N. Y.) at least three times before use. In the case of H-2 receptor patching and capping, treatment with only the primary antibody (mouse anti-H-2 antibody) with-

out adding a secondary antibody (anti-antibody, e.g., goat anti-mouse Ig) induces only 10–20% capped cells in the total thymocyte population. The proportion of patched and capped cells is enhanced to a maximum of 50–60% of the total population if the cells are treated with both primary and secondary antibodies (8, 18, 25). Consequently, a double-antibody treatment (i.e., mouse anti-H-2^b antibody and goat anti-mouse antibody), designated anti-anti-H-2 antibody, was used throughout all the experiments.

To demonstrate surface H-2 antigen patching and capping using the standard immunofluorescence technique, washed cells (1 × 10^7 cells/ml) were first treated at 0°C for 30 min with 100 μg/ml of mouse anti-H-2^b haplotype antibodies (a gift of Dr. Robert Hyman, The Salk Institute) which react with all the H-2 surface determinants. This was followed by treatment with 100 μg/ml of fluorescein-conjugated goat antibodies against mouse Ig for another 30 min at 0°C. Cells were then washed three times with RPMI 1640 medium to rinse off unbound immunoreagent. One portion of the cell suspension was continuously kept at 0°C and another portion was incubated at 37°C for 30 min to induce patching and capping. Labeled cells were examined with a Zeiss fluorescence microscope using a × 40 oil immersion lens with an epi-illuminator and photographed on Kodak Plus-X film.

When cells labeled with the mouse anti-H-2^b and fluorescein-conjugated goat anti-mouse Ig are kept at 0°C, surface H-2 antigens appear to be distributed uniformly over the cell membrane as shown in Fig. 1 a. However, if the immunolabeled cells are incubated at 37°C for 30 min, the H-2 antigens are induced to form aggregates on the cell surface which are typical of patching and capping as previously reported (8, 18, 25) (Fig. 1 b).

30 min of incubation at 37°C was found to induce the maximum percentage of cells exhibiting cap structures (50–60%). Longer incubation at 37°C leads to a decrease in the number of capped cells. This is most likely due to shedding and/or internalization processes. As shown in Fig. 1 b, at the time of maximum cap formation there are always some cells which display patch structures. Although the maximum amount of patching occurs before the maximum amount of capping in this system, it is clear that these two processes are rather asynchronous and significantly overlap each other in time. This type of behavior has been
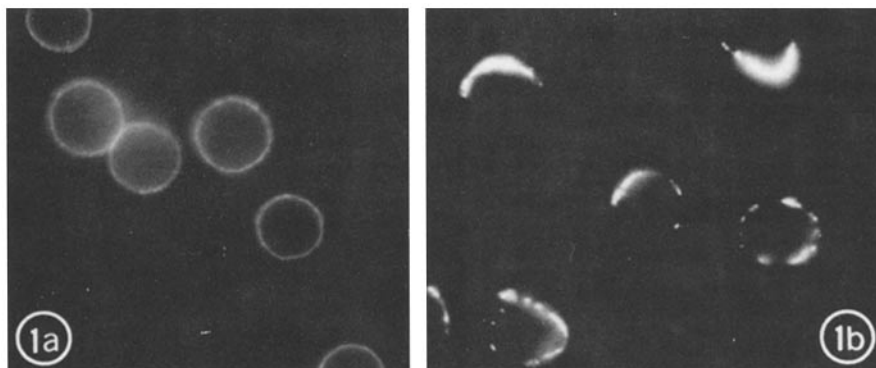
FIGURE 1 Immunofluorescent staining of surface *H-2* antigens on mouse thymocytes. (*a*) Uniform distribution on ring form: Cells were labeled with mouse anti-*H-2*[b] antibody plus fluorescent goat anti-mouse Ig at 0°C as described in the text. × 1,300. (*b*) Patches and caps: Cells were labeled with the same immunoreagent as in Fig. 1*a* except the cells were incubated at 37°C for 30 min. × 1,300.

reported by others (8, 18, 25). Therefore, under the present conditions one is forced to study a mixture of the two kinds of surface distributions.

In this paper, we shall describe the development of a novel approach using immuno-LPO conjugates to identify specific membrane proteins which are in close proximity to a particular antigen (in this case, the mouse *H-2* antigen) during antibody-induced patching and capping.

Rationale for Immuno-LPO-Labeling Procedure

According to the currently accepted fluid mosaic model (17), membrane proteins generally are located randomly throughout the plasma membrane as illustrated in Fig. 2*A*. Iodination by free lactoperoxidase has been commonly used for labeling a large variety of the surface membrane-associated proteins which have accessible tyrosine groups (6). The strategy behind using immuno-LPO conjugates is to restrict LPO molecules to specific locations on the membrane where they are able to catalyze the iodination of only those tyrosine-containing proteins which are in the immediate vicinity of the antibody-antigen complexes. As shown in Fig. 2*B*, surface *H-2* antigens bound with LPO-conjugated anti-anti-*H-2* antibody at 0°C are arranged in a random distribution pattern. In this situation the predominant iodination products should be the *H-2* antigen, the antibody molecules, and possibly any surface protein(s) always closely associated with the *H-2* antigen. However, when the immuno-LPO-labeled cells are incubated at 37°C for 30 min and form patches and caps, any proteins which co-aggregate with the *H-2* antigens

are potentially available for iodination. Other membrane proteins may be specifically excluded outside the *H-2* caps and, therefore, should not be labeled at all (Fig. 2*C*). Experimental tests supporting these predictions are described below.

Immuno-LPO Conjugation Technique

To chemically cross-link LPO with antibody molecules, a modification of the conjugation method developed by Ternynck and Avrameas (20) was employed. Goat antibody raised against mouse immunoglobulin (20 mg) was first dissolved in 3 ml of 0.1 M phosphate buffer, pH 6.0, plus 0.15 M NaCl (PBS). A 0.5-ml solution of *p*-benzoquinone (Eastman Kodak) at 40 mg/ml in ethanol was added to the antibody solution and incubated in the dark at room temperature for 1 h. The solution, which became brown in color, was then filtered through a 10-ml Sephadex G-25 column equilibrated with 0.15 M NaCl. The protein peak (OD 280) which emerged at the void volume was pink in color and contained the "activated antibody." 20 mg of LPO (Sigma Chemical Co., St. Louis, Mo.) was then added to the activated antibody solution, followed by ¹⁄₁₀ vol of 1 M bicarbonate buffer (pH 9.0). After an 18-h incubation at room temperature, the reaction was stopped by the addition of ¹⁄₁₀ vol of 1 M lysine (pH 7.5). The mixture was then passed through a Sephacryl S-200 (Pharmacia Inc., Piscataway, N. J.) column (4 × 80 cm) which was equilibrated and eluted with PBS (0.1 M phosphate buffer, pH 7.0, plus 0.15 M NaCl). The LPO-conjugated goat anti-mouse immunoglobulin eluted at the void volume, whereas the free LPO and unconjugated
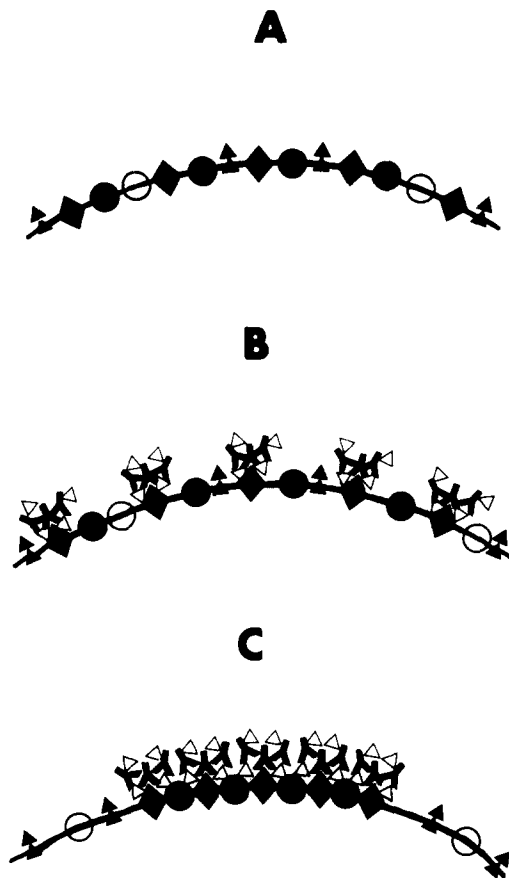
A

B

C

FIGURE 2 Schematic diagram of a plasma membrane treated and untreated with the immuno-LPO complex (LPO-anti-anti-*H-2*). (*A*) Typical fluid mosaic membrane with proteins (symbolized as ◆, ●, ○, ✦) randomly located throughout the plane of the membrane. (*B*) *H-2* molecules (◆) are bound at 0°C with their specific primary antibody (mouse anti-*H-2* antibody: ⅄) plus the LPO-conjugated goat anti-mouse antibody (LPO molecules: △; goat antibody: ⅄). The immuno-LPO complex should iodinate only the immunoglobulins, the *H-2* antigen, and possibly any surface protein always closely associated with the *H-2* antigen. (*C*) *H-2* antigens are induced to form patches and caps by incubation with the LPO-anti-anti-*H-2* complex at 37°C. In addition to the immunoglobulins and the *H-2* antigen, the immuno-LPO complex should iodinate some proteins (●) which are co-capping with the *H-2* antigen. Other membrane proteins (○, ✦) may be excluded from the *H-2* cap.

goat anti-mouse Ig were included on the column. Under the conditions described, ~10–20% of the LPO and the antibody remained unconjugated.

p-Benzoquinone was chosen as the cross-linking reagent instead of the conventional reagent, glu-

taraldehyde, since it has been found to better preserve both the immunological capability and the enzymatic reactivity of immuno-LPO conjugates (20). This particular method can be used to conjugate a large variety of different molecules as long as they have amino and either thiol or phenolic groups available (9, 10).

Patching, Capping, and Iodination by Immuno-LPO (LPO-Anti-Anti-H-2) Complex

To induce *H-2* patching and capping, 10^8 thymocytes were washed with RPMI 1640 medium three times and treated with 0.5 ml of mouse anti-*H-2*^{b} (100 μg/ml) at 0°C for 30 min. The cells were washed again with RPMI 1640 medium and incubated with 1 ml of the LPO-conjugated goat anti-mouse antibody (200 μg/ml) for an additional 30 min at 0°C. One portion of the immunolabeled cells was subsequently incubated at 37°C for 30 min while another portion of the sample was kept continuously at 0°C. As a control, normal mouse Ig was substituted for the mouse *H-2* antibody. After immunolabeling, the cells were washed twice with 5 ml of 20 mM glucose in PBS (pH 7.0) and resuspended in 1 ml of the same buffer. 200 μCi of ^{125}I-Na (ICN Nutritional Biochemicals, Cleveland, Ohio) and 1 U of glucose oxidase (Sigma Chemical Co.) were then added to the cell suspension to initiate the iodination reaction. In addition, a small aliquot of the cells was not treated with the immuno-LPO reagents, but was uniformly labeled using free LPO (200 μg/ml), ^{125}I-Na, and glucose oxidase. All of the iodination reactions were carried out at 0°C for 30 min and terminated by washing cells with an excess amount of RPMI medium at least five times. The iodinated cell suspensions, which incorporated ~350 cpm/μg of total protein, showed 90–95% viability as determined by trypan blue exclusion. The cells were examined both in the light microscope using an indirect immunofluorescence technique and in the electron microscope with autoradiography (EM-ARG).

For the immunofluorescence analysis, an aliquot of the iodinated cells was first fixed with 2% paraformaldehyde for 30 min at room temperature in 0.1 M phosphate buffer, pH 7.0. They were then labeled with fluorescein-conjugated rabbit antibody raised against goat immunoglobulin (100 μg/ml, 30 min at room temperature) to detect the

distribution of the LPO-anti-anti-*H-2* complex. The results shown in Fig. 3 indicate that the immunocomplex forms a fairly uniform pattern (ring forms and some micropatches) on the surface of cells kept at 0°C (Fig. 3 *a*), but will redistribute into typical patches and caps when the cells are incubated at 37°C for 30 min (Fig. 3 *b*). It is evident from the fluorescence pattern that the large majority of immunoreagent is closely associated with the cell surface and is not internalized.

Confirmation of the fact that the immuno-LPO complexes induce normal patches and caps on the surfaces of mouse thymocytes was obtained by examination of the cells using EM-ARG. In these experiments, an aliquot of the iodinated cells was immediately fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.0), postfixed with 2% osmium tetroxide in the same buffer, and then dehydrated with a series of ethanol solutions. Subsequently, cells were embedded in Epon and cut into thin sections which were exposed to Kodak photographic emulsion (type 129-01) for autoradiographic analysis according to the procedures previously published (15). The iodinated sections were developed after at least 1-mo exposure and examined in a Philips 301 electron microscope.

As shown in Fig. 4 *a*, silver grains are often found in small clusters which are fairly well dispersed on the surface of the thymocytes incubated with the LPO-anti-anti-*H-2* complex at 0°C. These small clusters of grains may indicate "micropatch" formation. In the case of cells treated with the immunoreagents at 37°C, a large majority of the silver grains are observed clustered into distinct caps or patches (Fig. 4 *b–d*). The iodination products are preferentially localized on the cell surface. However, there does appear to be a small amount of internalization of the *H-2* antigen representing only ~10-15% of the total silver grain count. The internal silver grains are more readily observed on the unstained EM-ARG sections shown in Fig. 4 *a* and *b*. In addition to internalization, we have detected a small amount of shedding or release of the immuno-LPO complexes (~10-15%) into the medium during the 30-min incubation at either 0° or 37°C. This determination was made on immuno-LPO complexes labeled with ^{125}I using the self-iodination procedure described in the next section. Finally, in control samples which were originally treated with normal mouse immunoglobulin in place of the anti-*H-2* antibody, no immunofluorescent staining nor silver grains were observed associated with the cells.

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis of Iodinated Thymocyte Surface Proteins

After iodination by either the immuno-LPO complex or free LPO as described above, a portion of the cells was solubilized with 3% SDS in the presence of 0.02 M Tris buffer (pH 7.0) and 0.1 M dithiothreitol by boiling at 100°C for 3 min. These iodinated samples were electrophoresed on a 12% acrylamide-*N,N'* methylene-bisacrylamide (wt/wt 30:0.4) slab gel using Neville's buffer system (12). All gels were stained with 0.1% Coomassie brilliant blue, destained with 30% methanol plus 7% acetic acid, dried under vacuum, and exposed for 3-5 d to Kodak X-ray film for detection of ^{125}I-labeled proteins.

Using free LPO to iodinate all available thymocyte surface membrane proteins, we have obtained the SDS-PAGE pattern shown in Fig. 5 *A*. There are six polypeptides which are extensively labeled. These bands, numbered *1-6*, have mol wt of ~200,000, 160,000, 60,000, 35,000, 25,000, and 12,000 daltons, respectively. In addition, there are a large number of other minor bands, particularly in the high molecular weight region.

When the thymocytes are treated at 0°C with LPO-conjugated anti-anti-*H-2* antibody, the iodination pattern (Fig. 5 *C*) shows just three major bands. These bands have been identified as the heavy and light chains of goat and/or mouse Ig and the *H-2* antigen. The identification of the Ig chains is based on the fact that purified goat as well as mouse Ig migrate on the gels at exactly the same rate as the two bands with mol wt of ~55,000 and 23,000 daltons. The *H-2* antigen band (mol wt 43,000 daltons) was identified by conventional immunoprecipitation techniques as described previously (21). Besides these three major bands, there are only two or three other minor bands which are distinguishable. Therefore, it appears that the immuno-LPO is primarily labeling only a few polypeptides localized in the very immediate vicinity of the antigen-antibody complex. There is very little labeling of other prominent membrane proteins under these conditions even though the *H-2* antigen is distributed almost uniformly over the cell surface (Figs. 3 *a* and 4 *a*). It is worth noting, however, that one of the observed minor bands appears to be migrating at the same position as band 1 (Fig. 5 *A*) which is the polypeptide most intensely labeled by free LPO.

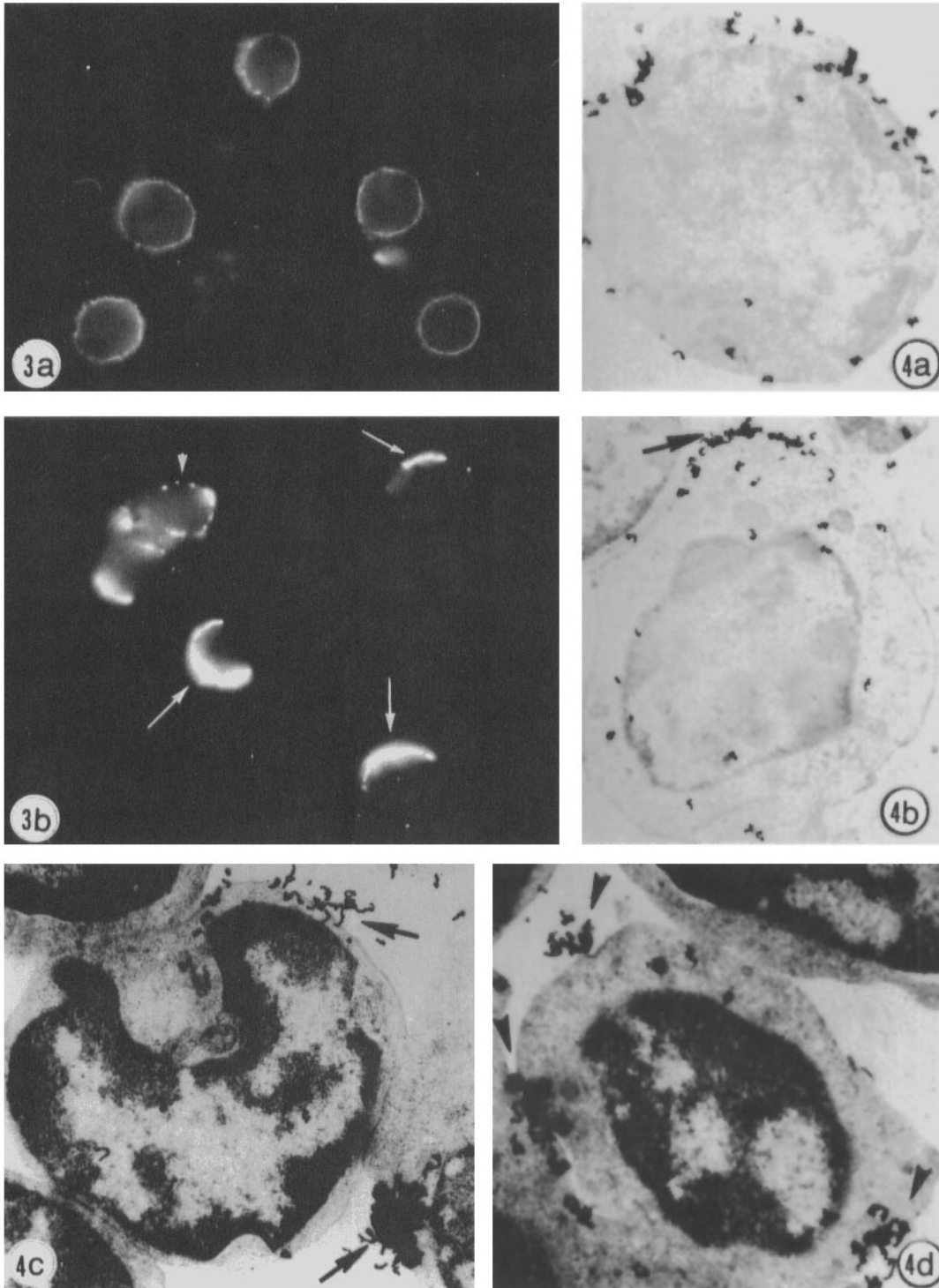After incubation of the cells with the immuno-

FIGURE 3   Immunofluorescent staining of thymocytes treated with the LPO-anti-anti-*H-2* complex. Cells were incubated for 30 min at either 0°C (*a*) or 37°C (*b*) with the immuno-LPO complex, carried through the iodination procedure at 0°C, and finally fixed and treated with fluorescein-conjugated rabbit anti-goat IgG to localize the immuno-LPO complex. (Arrows indicate capped regions.) × 1,300.

FIGURE 4   E.M.-ARG of thymocytes treated with the LPO-anti-anti-*H-2* complex. Cells were incubated for 30 min at either 0°C (*a*) or 37°C (*b–d*) with the immuno-LPO complex, fixed, and processed through the standard Epon thin sectioning procedures followed by autoradiography. *a* and *b* are unstained sections; *c* and *d* are stained with uranyl acetate and lead citrate. (Complete arrows indicate capped regions; arrowheads indicate patched regions.) × 7,200.
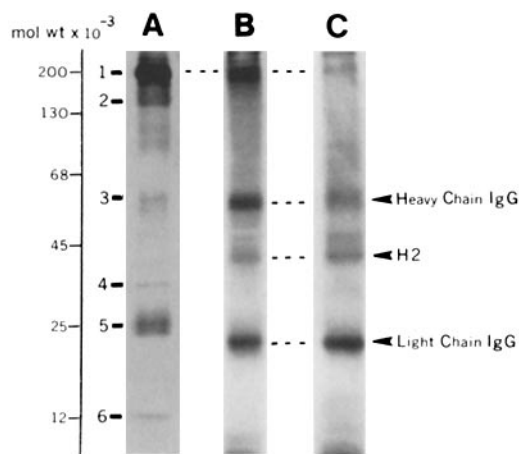
FIGURE 5 SDS-PAGE analysis of iodinated thymocyte surface membrane proteins. The 12% acrylamide gels were run with Neville's buffer system as described in the text. (A) Iodination by free LPO. (B) Iodination by the immuno-LPO complex after incubation at 37°C to induce patching and capping. (C) Iodination by the immuno-LPO complex at 0°C to prevent patching and capping. All iodination reactions were performed at 0°C. After solubilization of the cells, ~100 µg of total protein (~35,000 cpm) was loaded per well.

LPO complex at 37°C for 30 min to induce patching and capping, iodination was carried out at 0°C as before and the labeled polypeptides were displayed by SDS-PAGE autoradiography (Fig. 5 B). One observes that, again, the heavy and light chains of Ig and the H-2 antigen are prominently labeled. However, there is now an additional major band, apparently located at the same position (200,000 daltons) as band 1—the major surface protein iodinated by free LPO. Presumably, this 200,000-dalton protein is closely associated with H-2 antigen patch and cap structures.

Of the five other major bands identified in Fig. 5A, bands 4 and 6 do not appear in Fig. 5 B and C; bands 2, 3, and 5 may or may not be labeled. Further evidence for site-specific labeling by the anti-H-2 immuno-LPO complex was obtained by measuring the proportion of ^{125}I incorporated into the H-2 band by the immuno-LPO complex (at 0° and 37°C) and by free LPO. This was accomplished by slicing up the SDS polyacrylamide gels (identical to those shown in Fig. 5) and counting each slice for ^{125}I radioactivity. It was found that relative to the total counts on each gel, the H-2 region is at least three- to fourfold more intensely labeled by the immuno-LPO complex (Fig. 5 B and C) than by the free LPO (Fig. 5A).

As a control experiment to confirm which of the major bands in Fig. 5 B and C represent cellular surface proteins and which are derived from the immuno-LPO complex, the LPO-anti-anti-H-2 complex was allowed to self-iodinate at 0°C for 30 min in the absence of any cells. The standard reaction conditions were used, except the glucose oxidase and glucose were replaced with 0.03% H_2O_2. The iodinated products were precipitated by 10% TCA, washed with 90% acetone, and then solubilized for analysis of SDS-PAGE and autoradiography. Only two major bands (at the positions of the heavy and light chain IgG's) and one minor band (migrating with a mol wt of ~100,000 daltons) are apparent. Since there are no other bands visible—particularly in the 200,000- or 40,000- to 45,000-dalton regions—the 200,000- and 43,000-dalton bands found in Fig. 5 B and C are in all likelihood cellular surface proteins.

DISCUSSION

If cells are induced to form patches and/or caps and then treated with free LPO, the surface iodination patterns as analyzed by both EM-ARG and SDS-PAGE are found to be essentially identical to those obtained from cells without any patches and/or caps (data not shown). Consequently, we have developed a new labeling technique using immuno-LPO complexes which is able to specifically detect proteins present in the surface patch or cap structures.

The results of our initial studies on the patching and capping of mouse thymocyte H-2 antigens are presented as an example of the use of this novel labeling technique. At 0°C when the H-2 molecules are uniformly distributed over the cell surface, immuno-LPO catalyzed the iodination of primarily only three polypeptides: the heavy and light chains of Ig and the H-2 antigen itself. Most interestingly, after formation of H-2 patches and caps by incubation at 37°C, we have detected the presence of an additional surface polypeptide (band 1) in the immediate vicinity of the immuno-LPO complex. Two other surface polypeptides (bands 4 and 6) are not apparently labeled under these conditions. This technique allows one to identify those polypeptides which are specifically included in, or excluded from, the patch and cap structures.

Because of their similar mol wt (~200,000 daltons) and relative abundance, band 1 (Fig. 5A and B) is most likely the T200 protein shown by Trowbridge et al. (21) and Trowbridge and Mazauskas

(22) to be one of major T-lymphocyte glycoproteins. This protein appears to be intimately associated with H-2 patches and caps. It is noteworthy that we have previously demonstrated, using a double immunofluorescence technique, that T200 molecules co-cap with H-2 antigens in a T-lymphoma cell line (4). Further investigation of the role that this major lymphocyte surface protein plays in the patching and capping processes should provide significant information on the molecular mechanisms involved in ligand-induced receptor mobility, in general, as well as H-2-controlled immune responses and lymphocyte cell-cell interactions.

It has been found that β_2-microglobulin is associated in part with H-2 molecules in the lymphocyte membrane (16, 26). However, under our immuno-LPO labeling conditions, very little β_2-microglobulin (mol wt: ~12,000 daltons) appears to be iodinated either when H-2 antigens are uniformly distributed or in a patching/capping configuration. One obvious explanation for this result is that β_2-microglobulin does not have available tyrosine residues.

In conclusion, we believe that this new surface iodination method using immuno-LPO conjugates should be able to selectively label membrane polypeptides closely associated with any particular patch/cap structure. For the first time, it is now possible to carry out direct biochemical analysis of the membrane protein constituents involved in any ligand-induced receptor redistribution. Further studies on the polypeptides found in various ligand-induced patch and cap formations on both lymphoid and fibroblastoid cells are currently in progress.

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