Chapter 4

Patching and Capping: Plasma Membrane Protein Redistribution

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Introduction

One of the major concepts in cell biology is the idea that the fluid mosaic membrane and the cytoskeleton are an integrated, supramolecular structure. This complex is important for both cellular response to extracellular signals and the ability of the cell to interact with the extracellular milieu. The existence of this functional supramolecular complex was first demonstrated by studying the response of B lymphocytes to treatment with anti-immunoglobulin antibodies. The crosslinking of the cell surface immunoglobulin molecules causes a passive patching or clumping of the proteins in the plane of the membrane. This is then followed by a metabolism-dependent collection of the patches to one end of the cell (a process called capping). This lab exercise duplicates the original experiments on this phenomenon (Taylor et al. 1971). It demonstrates two major conceptual points: 1. the fluidity of the membrane and 2. the shift from passive diffusion (which leads to patching) to a metabolically active process (which leads to capping). This exercise teaches the basics of immunofluorescent staining, the use of metabolic inhibitors to distinguish active from passive processes, and the design of a controlled set of experiments to yield an unambiguous result.

Student Outline

Introduction

The plasma membrane consists of a fluid, lipid bilayer with protein molecules embedded in it. (See Alberts et al., Chapter 10.) The mobility and location of these proteins in the membrane are important factors in the functioning of the membrane and, therefore, in the functioning of the cell. This experiment demonstrates how one membrane protein changes its distribution in the membrane when it is bound by a ligand.

B-lymphocytes are the subclass of lymphocytes that produce and secrete immunoglobulins. They also have a membrane bound form of immunoglobulin expressed on their cell surfaces (IgM). The membrane bound IgM can be detected by incubating the cells with a fluorescently labeled antibody against IgG and visualizing the bound fluorescence using a fluorescence microscope. The anti-IgG will recognize the half of IgM molecule that does not undergo type switching during maturation. (See Alberts et al., Chapter 23.) Because antibodies are bivalent, they can cross-link the cell surface immunoglobulins by binding a different cell surface IgM molecule to each of the two antigen binding sites. The cell responds, in turn, by redistributing the cross-linked cell surface immunoglobulins. You will be using a combination of treatments to determine:

(a)What are the distribution patterns of IgM on normal and antibody-treated cells.

(b)What changes in distribution require metabolic energy from the cell, and what changes occur passively and are due only to the cross-linking.

Metabolism is inhibited either by treatment with sodium azide or by incubation at 4°C. By treating cells with fixative before adding the fluorescent antibody, you can determine what the normal, unperturbed immunoglobulin localization is.

Materials

On Lab Bench (lined with bench top cover)
- Micropipettors (1 set per group; 0.5-5 ml, 200-1000 µl, 50-200 µl, 5-50 µl)
- disposable pipette tips
- waste container for used tips
- 10-ml disposable beakers
- aluminum foil
- 5 microscope slides with frosted end (disposable)
- permanent marker (Sharpie)
- coverslips
- Pasteur pipettes
- Pasteur pipette bulbs
- glass waste container for used pipettes
- dissection tray (1 per group)
- dissection scissors (1 pair per group)
- probe (1 per group)
- medium sized Petri dish (60 x 15 mm; 1 per group)
- stainless steel mesh screen (80 mesh, 45 gauge; 3" x 3"; 1 per group)
- spoonula (1 per group)
- bucket of ice (1 per group)
- waterbath (37°C)
- benchtop centrifuges (swinging bucket rotors are preferred)
- test tube rack (1-2 per group)
- 2 plastic 15-ml disposable, conical, clear test tubes with caps (per group)
- 5 plastic 5-ml disposable test tubes with caps (per group)
- 10-ml graduated cylinder (2 per group)
- Parafilm

On Lab Bench at side of room
- fluorescence microscopes
- surgical gloves (various sizes: small, medium, large, extra-large)
- mouse (*Mus musculus*; 1 per group)
- wash bucket with hot soapy water

Solutions (See Appendix A for sources.)
- Dulbecco's Minimum Essential Medium (MEM)
- Fixative - 10% formalin in Dulbecco's phosphate buffered saline (1X)
- 0.1M sodium azide
- fluorescein-labeled rabbit anti-mouse IgG
- Dulbecco's phosphate buffered saline (PBS) 1X
- 70% ethanol

Safety Notice: Formalin is a solution of formaldehyde. Formaldehyde produces irritating and toxic vapors and should only be handled under a fume hood. The capped tubes may be kept on the benchtop. Sodium azide is highly toxic; it inhibits electron transport chains, in a manner similar to cyanide. It should be handled with care. Azide can also form explosive complexes with some metals in sink drain pipes Check with your institutional safety office for local requirements for disposal.

Before the lab

Read over the procedure and the flow chart. Make sure that you understand the reason for the five different treatments, and how comparing results from the different treatments gives you information about the way molecules redistribute in the membrane.

Laboratory Procedures

1. Sacrifice one mouse (this will be done for you). Remove the spleen and place it in 10 ml Dulbecco's Minimum Essential Medium (MEM). The spleen is on the left side of the mouse, just under the lower margin of the ribs. It is a dark red organ, approximately 1.5-2 cm in length and 0.5 cm in diameter. To remove the organ, wet down the mouse's fur with 70% ethanol. Use scissors to open an incision along the left side of the abdomen up through the lower ribs. Lift the spleen away from the other viscera and trim away the fat and connective tissue.
2. Transfer the spleen to the surface of a stainless steel mesh screen. Wet it with a few drops of MEM and force it through the mesh, using the flat surface of a spoonula, into 10 ml MEM in a Petri dish underneath the mesh. Rinse the solution through the screen several times to rinse off cells trapped in the mesh.
3. Transfer the resulting cell suspension from the Petri dish into a 15-ml disposable plastic test tube (clear with conical bottom) with cap and place on ice.
4. Let the cell suspension stand for 3 minutes. Transfer the upper 9 ml of the cell suspension to a fresh 15 ml tube, leaving the fast-settling debris in the bottom 1 ml.
5. Centrifuge the tube for 3 minutes, at speed 5 (approximately 1229 x *g*), in a benchtop clinical centrifuge.
6. Discard the supernatant and resuspend the pellet in 5 ml of Dulbecco's MEM. Mix by firmly tapping the tip of the tube on the bench top, or vigorously slapping the tube against the heel of your hand. Do not vortex. Keep the tube on ice.
7. Pipette 5 equal 1 ml aliquots of the cell suspension into five 5-ml disposable plastic test tubes with caps. Pellet the cells with a 3 minute spin, at speed 5 (approximately 1229 x *g*), in the benchtop clinical centrifuge.
8. Discard the supernatant solution and resuspend each pellet in 25 µl Dulbecco's MEM. Incubate the tubes on ice at least 5 minutes.

Carry out the next three steps (9-11) in a fume hood.

9. To one of the five test tubes, add 500 µl of fixative [10% formalin in Dulbecco's phosphate buffered saline (PBS)]. Let the sample sit 15 minutes on ice. This will fix the sample.
10. Wash the cells twice by pelleting 3 minutes at speed 5, decanting the supernatant solution, and resuspending in 1 ml Dulbecco's MEM. After the last wash, resuspend the pellet in 25 µl MEM.
11. To another two of the five test tubes, add 10 µl 0.1 M sodium azide.
    Note: Those tubes requiring Na^+ azide should receive it at least *10 minutes before* the fluorescein-labeled rabbit anti-mouse (FITC) antibody is added. Also, the FITC should be precooled *before* it is added to the tubes.
12. To all five of the test tubes, add 25 µl ice-cold fluorescein-labeled rabbit anti-mouse IgG (FITC).
13. Take two of the test tubes of cells, one with sodium azide and one without, and warm them to 37˚C in a waterbath for 15 minutes. Keep the other three test tubes on ice.

14. After 15 minutes, add 1 ml of ice cold fixative to all the samples. Incubate the samples on ice for another 15 minutes.
15. Pellet the fixed cells in the centrifuge, as above. Discard the supernatant solutions. Wash the cells twice by resuspending cells in 1 ml PBS and then pelleting them in the centrifuge. After the last wash, resuspend the pelleted cells in 50 µl PBS.
    At this point the fixed cells can be stored in a refrigerator for up to several days before scoring without significant degradation in the quality of the result.
16. Examine cells from each of the five different test tubes with a fluorescent microscope. Put approximately 10 µl of a cell suspension on a microscope slide under a 22 X 22 mm coverslip. Examine the cells with a fluorescent microscope. Count 40-50 fluorescently stained cells. Categorize the immunoglobulin groupings on the cells' surfaces into patches, caps, and ring staining.

What to look for

Under the fluorescence microscope, you should see a small proportion of cells stained with the fluorescent antibody, exhibiting a green glow. Those cells will have one of three fluorescence patterns:

1) Ring staining - a uniform staining around the entire periphery of the cell.
2) Patched - several brightly staining small patches randomly distributed about the cell's periphery.
3) Capped - the fluorescence is localized at one end of the cell, forming a "cap".

Data Treatment

Count 40-50 fluorescently stained cells from each of your five samples. Categorize the slide samples into the three classes listed above. Discuss how the pattern of fluorescence depends on (a) the fluidity of the membrane and (b) normal energy metabolism. Why are only some of the lymphocytes stained with the fluorescent antibody?

Notes for the Instructor

This laboratory uses the spleen from a mouse as the source of cells. It is necessary to obtain approval from an institutional animal care committee prior to the use of animals in teaching labs. The fastest way of sacrificing the mouse is cervical dislocation, but an overdose of drugs or CO_2 is also acceptable. The animal should be sacrificed by qualified animal care personnel; students should be presented with a freshly killed animal (within 10-15 minutes of termination) for dissection.

Students must be told to focus through the cells at which they are looking. If they don't, they may conclude that a capped cell is a patched cell, or that a patched cell is a ring-stained cell, due to the random selection of the optical plane of the plasma membrane that is observed and/or the blurring in the medial focal plane of the cell. Students must also be reminded that both the fixative they are using and 0.1M sodium azide are toxic. Care must be taken with their use: gloves must be worn and the formalin must be kept in the fume hood.

Table 4.1. Table for Collecting Data.

Tube	Ring	Patch	Cap
#1			
#2			
#3			
#4			
#5			

Future Directions

The basic capping and patching experiment can form the foundation of a large series of experiments. More extensive experiments using a single technique or experimental system would allow the students to expend more effort on learning how to design experiments and less effort on learning a mass of new factual material every week.

1) The same experiment could be used as the basis for teaching electron microscopy techniques by using a ferritin-derivatized anti-IgG and processing the cells for EM rather than fluorescent light microscopy. This would allow the students to evaluate co-localization of cytoskeletal elements with the cell surface molecules.

2) The same experiment could be repeated with the addition of cytoskeleton-perturbing drugs, to help the students understand the use of specific drugs in evaluating cell biological mechanisms. Fluorescent phalloidin or anti-tubulin antibodies can also be used for double labeling and visualizing the cytoskeleton to supplement the drug perturbation studies.

3) Concanavalin A at different concentrations has been shown to alter the mobility of membrane proteins. The effect appears to be mediated via cytoskeletal interactions. A whole series of experiments using a combination of Con A and cytoskeleton-perturbing drugs could be designed by the students and executed over several lab periods.

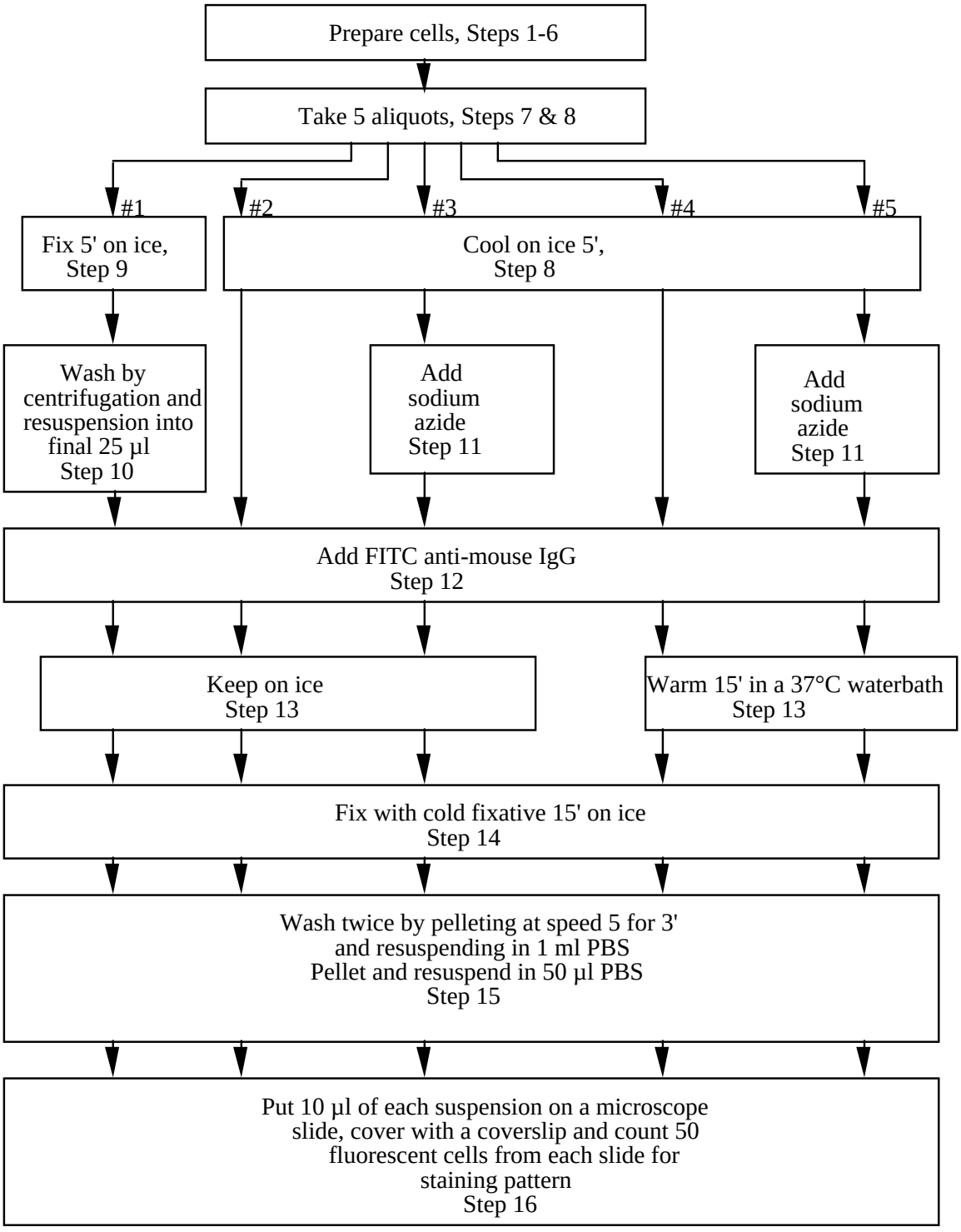

Figure 4.1. Flow chart of the patching and capping experiment.

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Appendix A
Preparator's Guide

Solutions

1. Dulbecco's minimum essential medium (Gibco catalogue #11965-050)
   -require approximately 30 ml per group
2. 10% formalin in Dulbecco's phosphate buffered saline (PBS)
   -require approximately 6 ml per group
   -for 100 ml; 10 ml 37% concentrated formalin, 10 ml Dulbecco's 10X PBS (see below), 80 ml double distilled, deionized water ($DDDH_2O$)
3. 10X Dulbecco's PBS (without Ca^{2+} and Mg^{2+}); pH should be 7.1-7.4
   -in 500 ml $DDDH_2O$

KCl	1.0 g
KH_2PO_4	1.0 g
NaCl	40.0 g
Na_2HPO_4	5.72 g

4. 0.1M Sodium Azide (Sigma catalogue #S-2002)
   Sodium azide is highly toxic. Wear gloves.
   -require 20 µl per group
   -0.065 g in 10 ml Dulbecco's 1X PBS = 0.1M Sodium Azide
   (NOTE: 10 ml Dulbecco's 10X PBS + 90 ml H_2O = Dulbecco's 1X PBS)

Antibodies

Fluorescein-labeled Rabbit anti-mouse IgG (Sigma catalogue #F-7506)

-1/10 dilution of stock from manufacturer = 25µl IgG into 225µl Dulbecco's 1X PBS. There is a wide range of dilutions that will work, but too high a concentration will not work well because the antibody will be in such excess that cross-linking will not occur. We typically buy one batch of FITC (fluorescein isothiocyanate labeled anti-mouse Ig) antibodies, test a series of dilutions, and then freeze away the aliquots of appropriate concentration and volume for several years worth of labs. Note: Although the cell surface immunoglobin is IgM, antibodies raised against IgG will react strongly with the light chains and the amino-terminal two heavy chain domains, which do not differ between IgG and IgM.
-require 250 µl per group; aliquot amounts for students in 1 ml polypropylene vials.
-Keep at -20°C prior to use.

Microscope

Any fluorescent microscope that will visualize fluorescein and with 20X and 40X objectives will suffice. The choice of fluorophore is not critical, so if the microscope available will only visualize other fluorophores, then an appropriate antibody change can be made.

Appendix B
Expected Results

Treatment 1 (immediate fixing with fixative): produces ring staining

Treatment 2 (no treatment with chemicals; on ice): produces predominantly patching

Treatment 3 (sodium azide on ice): produces predominantly patching

Treatment 4 (no treatment with chemicals; 37°C): produces capping

Treatment 5 (sodium azide at 37°C): produces predominantly patching

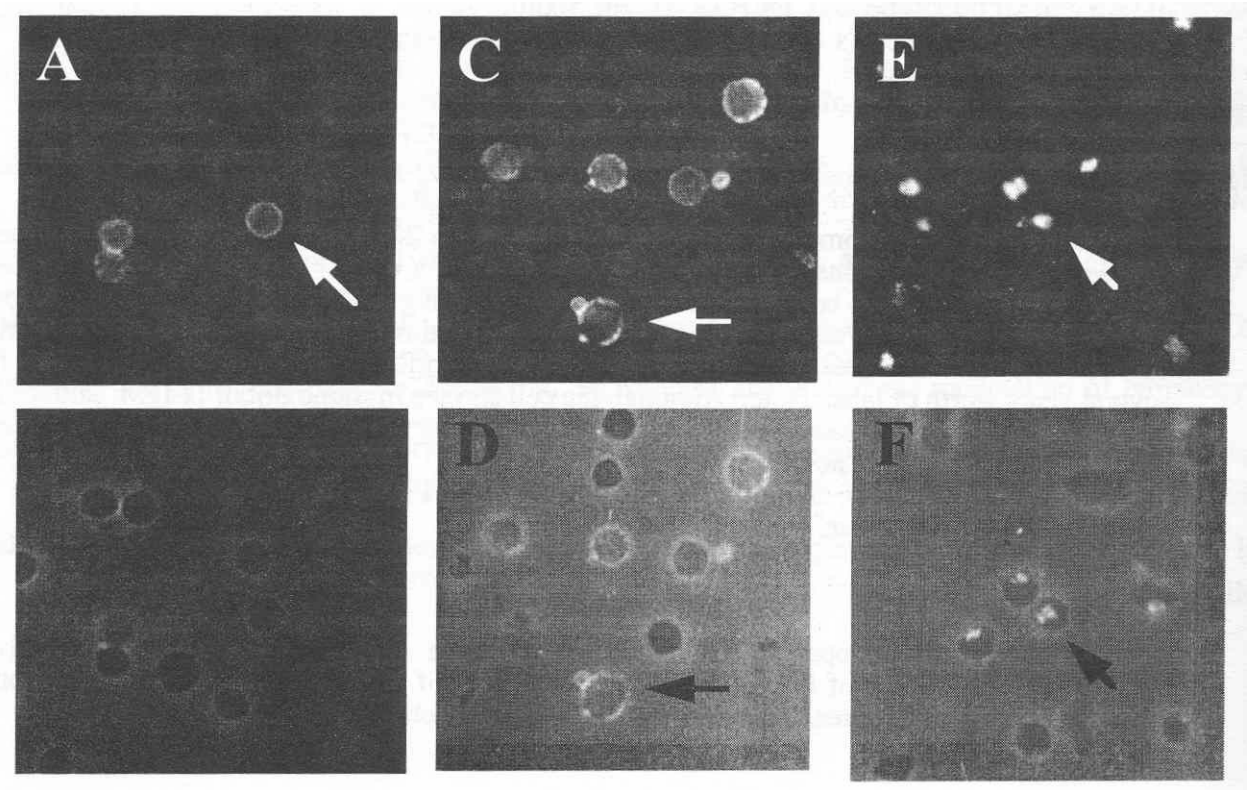

Figure 4.2. Fluorescence and phase contrast-fluorescence photographs of ringed (A, B), patched (C, D), and capped (E, F) staining patterns. Representative staining patterns of rings, patches, and caps are indicated by arrows. (Note: F is rotated 180° compared to E).