Cytoskeletal involvement in the sequential capping of rat thymocyte surface glycoproteins

CHRISTOPHER E. TURNER*, MARY R. NEWTON and DAVID M. SHOTTON†

Department of Zoology, University of Oxford, South Parks Road, Oxford OXI 3PS, England

* Present address: Department of Anatomy, University of North Carolina, Chapel Hill, NC 27514, USA
† Author for reprint requests

Summary

The independent capping of the three major rat thymocyte glycoproteins, the leucocyte-common (L-C) antigen, the leucocyte sialoglycoprotein (LSGP) and Thy-1, was investigated using specific monoclonal antibodies. The capping of each antigen did not require redistribution of the other major surface glycoproteins, and was accompanied by a partial co-capping of the cytoskeletal proteins fodrin and actin, but not of tubulin. A study of the ability of a cell that already possesses one glycoprotein cap to cap a second different glycoprotein showed that this was possible in all cases to varying degrees, the second cap always forming at the same position on the cell surface as the first. Colchicine failed to perturb this observed sequential capping polarity, indicating that microtubules did not direct this second capping event.

Key words: sequential capping, rat thymocyte, actin, fodrin, tubulin, cytoskeleton, membrane, glycoproteins.

Introduction

The capping of a variety of leucocyte cell surface glycoproteins is observed to occur after specific antibody cross-linking and incubation at 37°C, a process that has been shown to be energy dependent (Taylor et al. 1971) and that apparently involves the redistribution of a variety of cytoskeletal proteins, as shown by dual-label immunofluorescence techniques (Bourguignon & Singer, 1977; Singer et al. 1978; Heath, 1983; Levine & Willard, 1983; Bourguignon & Bourguignon, 1984). These observations have led to the proposal of a mechanism for capping that involves the association of the cross-linked surface glycoproteins with the underlying cytoskeletal actomyosin contractile machinery via a common 'protein X' (Bourguignon & Singer, 1977).

Following the discovery that the murine analogue of the leucocyte common (L-C) antigen (T-200) is intimately associated with the cytoskeletal actin-binding protein fodrin (Bourguignon et al. 1985), these workers proposed this protein as a candidate for protein X. For the L-C antigen to perform this role, it would be expected to redistribute during the capping of other antibody cross-linked glycoproteins. In this paper we report that the $180 \times 10^3 M_r$, rat thymocyte L-C antigen, defined by recognition by the NDS58 monoclonal antibody (Newton et al. 1986), is not redistributed during the capping of the leucocyte sialoglycoprotein (LSGP) or Thy-1 in rat thymocytes, and thus appears not to be involved in the capping mechanism. Elsewhere we describe another hitherto unidentified $205 \times 10^3 M_r$ glycoprotein, gp205, with properties resembling those expected of protein X (Turner & Shotton, 1987).

While the characteristics of capping of a single antigen have been thoroughly investigated and characterized (for review see Bourguignon & Bourguignon, 1984), few studies have been performed investigating the capping of a second antigen on cells already possessing an antibody-induced cap of a first antigen. Utilizing the fact that, once capped, the L-C antigen, the LSGP or the Thy-1 molecules of rat thymocytes remain on the cell surface rather than being endocytosed or sloughed off, we have studied the ability of a second antigen, suitably cross-linked with labelled antibody, to be sequentially capped to the same site on the cell surface as the first.
The role of microtubules in the capping process remains an area of controversy. Although Rogers et al. (1981) predicted that they performed an important role in directing a cap to a specific region of the cell surface, usually overlying the Golgi apparatus and microtubule-organizing centre (MTOC), as observed in splenic lymphocytes by De Petris & Raff (1972), Unanue et al. (1972), Stackpole et al. (1973) and De Petris (1975), their presence was found not to be essential for cap formation per se (Edelman et al. 1973; Rogers et al. 1981). In view of this proposed organizational role for microtubules, we have investigated the effect of disrupting the microtubules by colchicine treatment prior to the sequential capping of a second antigen on a cell already possessing one antigen cap, since in the absence of colchicine the second cap always forms in the same position as the first, implying that the cell possesses a strong capping polarity.

**Materials and methods**

**Thymocyte preparation**

The thymus was removed from an exsanguinated 6-week-old Lewis rat and washed in phosphate-buffered saline (PBS). The tissue was finely chopped with scissors in PBS and pressed through a fine-mesh stainless steel wire sieve using the plunger from a 5 ml plastic disposable syringe. The resulting cell suspension was filtered through a wisp of non-absorbent cotton wool to remove aggregates. The cells were then washed twice in PBS by centrifugation at 320 g for 8 min.

**Capping antibodies**

**Primary antibodies.** W3/13: the W3/13 mouse monoclonal antibody (IgG1), produced by immunization with rat thymocyte membranes, labels a monomorphic determinant on rat thymocytes, T lymphocytes (but not B lymphocytes), polymorphs and brain cells (Williams et al. 1977). It also labels plasma cells and myeloma cells (Brown et al. 1981). The antibody was shown to recognize a 95K (K = 10^3 M_γ ) glycoprotein-like protein on these cells, termed the leucocyte sialoglycoprotein (LSGP), using W3/13 affinity columns and immunoprecipitation (Standring et al. 1978).

**MRC OX-7:** the MRC OX-7 mouse monoclonal antibody (IgG1) was produced using purified thymocyte Thy-1 (M_1, 25×10^3) from deoxycholate-solubilized rat thymocyte membranes. It recognizes the Thy-1.1 determinant on rat thymocytes, B lymphocytes, bone marrow cells and brain cells (Mason & Williams, 1980).

**MRC OX-1:** purified rat thymocyte leucocyte-common (L-C) antigen was used to produce the MRC OX-1 mouse monoclonal antibody (IgG1) (Sunderland et al. 1979), which was found to recognize differing forms of the molecule on different lymphoid cell types (Sunderland et al. 1979; Standring et al. 1978). The thymocyte, T lymphocyte and B lymphocyte forms have been found to have molecular masses of 180K, approx. 200K (multiple bands) and 240K (broad band), respectively (Woollett et al. 1985). Ascites fluids containing W3/13, OX-1 or OX-7 were obtained from Serotec Ltd (Bicester, England). Alternatively, culture supernatants were obtained from hybridoma cells kindly donated by Dr A. F. Williams.

**NDS58:** the rat monoclonal antibody NDS58 (IgG2a) was produced by fusing the rat myeloma line Y3Ag1.2.3 with spleen cells from AS rats immunized with Lewis rat thymocytes. The antibody recognizes a glycoprotein of 65K (non-reduced) expressed on mouse T-cytotoxic and T-suppressor cells and thymocytes (Ledbetter & Herzenberg, 1979). It does not cross-react with rat thymocytes, as determined by fluorescence-activated cell sorter (FACS) analysis (data not shown). Culture supernatant containing NDS58 was obtained from hybridoma cells kindly provided by Dr B. Thomas.

**Secondary antibodies.** To induce capping and to label the antigen for indirect immunofluorescence, appropriate fluorescein isothiocyanate (FITC)- or tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG or goat anti-rat IgG antibodies (Sigma) were used, diluted between 1:25 and 1:66 in PBS. At least 60 min prior to use, 10% normal rat serum was included with the secondary antibodies, to block any non-specific cross-reactivity with rat proteins. This was substituted with 10% human AB serum when using NDS58 as the primary antibody, which proved equally effective in preventing background staining. To induce capping of the L-C antigen, additional unlabelled rabbit anti-mouse IgG (a kind gift from Dr K. J. Wood) was included with the fluorescently labelled secondary antibody.

**Labelling of cells**

A suspension of rat thymocytes was divided into samples containing 10^7 cells, each in a maximum volume of 100 μl in a 3 ml tube. A 100 μl portion of a 1:100 dilution of ascites fluid or 100μl of undiluted spent supernatant of primary antibody was added to the cells, which were then incubated for 60 min on ice with occasional shaking to maintain the cells in suspension. The cells were washed three times in PBS containing 0-1% bovine serum albumin (PBS-BSA) by centrifugation at 320 g for 8 min, and were then incubated in 100 μl of the appropriate secondary antibody for 60 min on ice. After incubation, excess antibody was removed by washing as described above.

**Induction of capping and fixation of cells**

Capping was induced by incubation of the labelled cells at 37°C, and was terminated at the required time by the addition of an excess of ice-cold PBS containing 1:5% formaldehyde. After fixation on ice for 30 min, the cells were pelleted by centrifugation at 320 g for 8 min and resuspended in 50 μl of PBS.
Preparation of cells for observation
A 7 μl sample of the suspended labelled cells was spread on to a subbed slide (i.e. a slide that had been dipped into warm 0-5 % gelatine, 0-125 % CrK(SO₄)₂·12H₂O, 0-01 % Teepol detergent, and then air dried), and gently warmed until almost dry over a small bunsen flame. (Since the cells were only lightly fixed, complete drying was avoided, as this resulted in the disruption of the cells and also in a reduction of the fluorescence signal.) The cells were then mounted under a sealed coverslip in 90% glycerol, 10% PBS and 2 μg ml⁻¹ Hoechst 33258 (Polysciences Inc.), and viewed by epifluorescence illumination using a Zeiss Standard 18 microscope fitted with narrow bandpass filter sets appropriate for FITC, TRITC and Hoechst. Results were recorded on Agfachrome RS1000 colour slide film at 1000 ASA using exposure times of between 15 and 30 s.

Determination of the distribution of other antigens on capped cells
The antigen to be capped was labelled with the appropriate monoclonal antibody and corresponding second layer, induced to cap by incubation at 37°C for a period predetermined to produce the maximum number of caps (see Results), and then placed on ice for 20 min. Labelling of the second antigen was performed at 0°C for 60 min, in the presence of 10 mM-sodium azide to inhibit oxidative phosphorylation and thus reduce the possibility of energy-dependent capping of the second antigen. Following this labelling, the cells were fixed in ice-cold PBS containing 1-5 % formaldehyde and mounted on slides for observation. To avoid cross-reactivity between the various antisera, each was blocked with human AB serum.

Concurrent capping of two antigens
To investigate the concurrent capping of two glycoproteins, both were labelled simultaneously, using the normal quantities of antibody for each, prior to incubation at 37°C.

Sequential capping of a second antigen
Following the labelling and capping of the first antigen, the second antigen was labelled as described in the previous section, but omitting the sodium azide. The cells were then incubated for a second time at 37°C to induce the capping of the second antigen, prior to fixation and mounting.

Fluorescence labelling of cytoskeletal elements in detergent-extracted cells
Fodrin staining. Capped or uncapped cells were fixed for 20 min at 0°C in PBS containing 1-5 % formaldehyde. The cells were washed in PBS and permeabilized by resuspension in 0-2 % Triton X-100 in PBS on ice for 20 min. Following pelleting by centrifugation, samples of the permeabilized cells were carefully dried onto subbed slides for immuno-labelling.

The permeabilized cells were incubated for 60 min at room temperature in 25 μl of a 1:10 dilution of rabbit anti-human brain fodrin antiserum, which has been shown by immunoblotting to cross-react with both the α (240K) and β (235K) polypeptides of brain fodrin, and with a single 240K polypeptide in rat thymocytes, presumed to be the α fodrin gene product (Fig. 1). After extensive washing in 0-1 % Triton X-100 in PBS, the anti-fodrin molecules were localized by incubating the cells for 60 min prior to observation in the following secondary antibody mixture: 1:50 TRITC–porcine anti-rabbit Ig (Miles), 5 % normal rat serum, 5 % normal mouse serum, and 0-1 % Triton X-100 in PBS. The normal rat and mouse sera were included to reduce the cross-reactivity of the anti-rabbit Ig with rat and mouse proteins.
F-actin staining. Cells, first fixed and permeabilized as described above for fodrin staining, were incubated in a 1:100 dilution of TRITC-phalloidin (Molecular Probes Inc., Oregon, USA; original concentration 100 unit ml⁻¹) for 10 min or longer (see Results), washed in PBS and mounted for observation.

Tubulin staining of unchallenged cells. In order to visualize the microtubule distribution, cells were fixed in 3-7% formaldehyde in PBS and permeabilized with 0.2% Triton X-100 in PBS. The cells were incubated with a 1:50 dilution of the rat anti-yeast tubulin monoclonal antibody YL1/2 (Kilmartin et al. 1982) kindly donated by Dr J. V. Kilmartin. After washing, the antibody was visualized using a second layer of a 1:40 dilution of TRITC-rabbit anti-rat Ig in PBS containing 10% human serum.

Since this monoclonal antibody was derived from a rat cell line, blocking of anti-rat activity of the second layer with rat serum could not be performed in the normal way. Consequently, as for the labelling of NDS58, the non-specific cross-reactivity of the second layer was blocked using human AB serum. The monoclonal rat anti-mouse Ly-2 antibody was used as a control. Fluorescently stained microtubules were photographed using Ilford HP5 black-and-white print film exposed at 800 ASA, with exposure times of between 8 and 20 s.

Effect of microtubules on the capping of a second antigen
To investigate the requirement for microtubules in the capping of a second antigen, the LSGP on cells was first induced to cap by addition of the appropriate antibodies. The cells were then placed on ice for 60 min to depolymerize their microtubules, and labelled with antibodies to the L-C antigen. The cells were incubated with each layer of antibody for 60 min at 0°C in PBS containing 10⁻⁴ M-colchicine. Removal of unbound antibody was also performed with PBS washes containing 10⁻⁴ M-colchicine. The L-C antigen was then induced to cap by warming to 37°C. At the required time intervals, cells were fixed in warm PBS containing 3-7% formaldehyde.

Results

Capping of individual antigens

The capping of the L-C antigen or the LSGP induced by specific monoclonal antibodies in the presence of fluorescently conjugated anti-immunoglobulins was, as expected, temperature dependent, the cells demonstrating staining over the entire cell surface when maintained at 0-4°C (seen as a ring of fluorescence of varying intensity when focusing at the equator of the cell, indicating some local patching) (Fig. 3A,C), and showing staining restricted to a cap of bright fluorescence at one pole of the cell after incubation at 37°C (Fig. 3B,D). The rates and extent of capping of each of the antigens varied. Capping of the LSGP under the conditions described in Materials and methods was very rapid, with 93% of cells possessing caps after 8 min at 37°C and reaching a maximum of 98% of cells with caps after 16 min (Fig. 2A). The capping of the L-C antigen using monoclonal NDS58 as the primary antibody, and with the additional inclusion of unlabelled rabbit anti-rat IgG in the second antibody layer, reached a maximum of 87% of cells possessing caps after 60 min at 37°C (Fig. 2B), while use of the monoclonal OX-1 as the primary antibody under similar conditions resulted in the capping of the L-C antigen on 83% of cells within 30 min (Fig. 2C).

The capping of Thy-1 was less straightforward, being very sensitive to the concentrations of the antibodies used. High concentrations of secondary FITC-goat anti-mouse IgG (e.g. a 1:33 dilution, as used for the capping of the LSGP) resulted in the complete immobilization of the Thy-1 antigen on the cell surface, after primary labelling with monoclonal OX-7. Lower concentrations led to the capping of a proportion of Thy-1 molecules on the cell surface, producing a concentrated region of fluorescence, while leaving a ring of uncapped Thy-1 fluorescence around the rest of the cell (Fig. 3F). For counting purposes these cells were deemed to have capped. Even under optimal conditions only 64% of the cells showed capped Thy-1 after 30 min at 37°C (Fig. 2D).

The caps of all three antigens appeared to remain on the cell surface after prolonged (2 h) incubation at 37°C, rather than being endocytosed, as is generally the case with capped surface Ig on B lymphocytes, or sloughed off into the surrounding medium. A secondary antibody layer was an absolute requirement for capping after primary monoclonal antibody labelling (data not shown). Cells challenged with 'irrelevant' control primary monoclonal antibodies were always totally unlabelled by the fluorescent second layer (Fig. 3G,H).

Independent capping

Cells labelled with the anti-L-C antigen antibody NDS58 were capped and subsequently relabelled at 0°C with antibodies directed against the LSGP or Thy-1. In the presence of the L-C antigen caps, the LSGP or Thy-1 molecules remained distributed over the entire surface of the cells, showing a typical ring-like staining pattern (Fig. 4A-D). Staining of L-C antigen capped cells with the control anti-spectrin monoclonal antibody 56A showed only faint background staining (not shown). Reciprocally, following the capping of the LSGP or Thy-1 antigen, the L-C antigen on the same cells revealed by NDS58 remained distributed over the entire cell surface, although on a minority of Thy-1 capped cells a little additional L-C antigen staining was observed coincident with the caps (Fig. 4E-H).
Fig. 2. Kinetics of capping of thymocyte glycoproteins. Samples of cells labelled with antibodies to the L-C antigen, the LSGP or Thy-1 and appropriate second layers (see Materials and methods) were induced to cap by warming to 37°C. At various time intervals the cells were fixed and prepared for immunofluorescence observation (see Materials and methods), and the number of uncapped (●●●●), patched (---) and capped (■■■■) cells was determined for: A, the LSGP antigen capped with W3/13 and FITC-goat anti-mouse IgG; B, the L-C antigen capped with NDS58 and TRITC-rabbit anti-rat Ig; C, the L-C antigen capped with OX-1 and FITC-goat anti-mouse Ig; and D, Thy-1 capped with OX-7 and FITC-goat anti-mouse IgG. At each time point at least 200 cells were counted, cells with overall diffuse fluorescence giving continuous ring staining around the cell perimeter being scored as uncapped, those with discontinuous fluorescence localized to at least two regions being counted as patched, and those with fluorescence emanating from a single region occupying less than half of the cells' surface area being classed as capped.

Concurrent capping
Cells labelled simultaneously with antibodies to the L-C antigen and the LSGP, or to the L-C antigen and Thy-1, exhibited ring-like staining for both antigens when maintained at 0–4°C. Upon incubation at 37°C the L-C antigen and LSGP on cells labelled with antibodies to both of these antigens, were observed to cap concurrently to the same position on the cell surface. The fluorescein and rhodamine staining, corresponding to the position of the two antigens, was co-extensive at all stages of the capping process (results not shown).

In contrast, incubation at 37°C of cells labelled for both the L-C antigen and Thy-1 resulted in only partial capping of both antigens to the same place, with significant ring staining around the periphery of the remainder of the cell (Fig. 5A,B).

Sequential capping
Cells with capped LSGP were subsequently labelled with antibodies to the L-C antigen. Incubation of these cells at 37°C caused the L-C antigen to form a cap in the same position on the cell surface as the already-formed LSGP cap. Observations of the L-C antigen during the early stages of its capping indicated that the L-C antigen capped progressively towards the pre-existing LSGP cap, rather than forming a cap in a...
Fig. 3. Uncapped and capped distribution of rat thymocyte glycoproteins. In this and the subsequent figures, cells were labelled with a specific monoclonal antibody and the appropriate FITC- and TRITC-conjugated second antibody layer (green and red fluorescence, respectively), as described in Materials and methods. Capping, where it was performed, was induced by incubation at 37°C for the period necessary to induce maximum capping (see Fig. 2) unless otherwise stated, and the cells were then fixed and prepared for double-labelling immunofluorescence colour photomicrography, using the appropriate Hoechst, FITC or TRITC filter combinations (see Materials and methods). A. Uniform distribution of the L-C antigen following labelling with NDS58 at 0°C. B. The NDS58-labelled L-C antigen showing a capped distribution after subsequent incubation at 37°C. C. Uniform distribution of the LSGP following labelling with W3/13 at 0°C. D. The W3/13-labelled LSGP showing a capped distribution after subsequent incubation at 37°C. E. Uniform distribution of Thy-1 following labelling with OX-7 at 0°C. F. OX-7-labelled Thy-1 showing a partially capped distribution after subsequent incubation at 37°C. G. Cells labelled with control mouse monoclonal anti-spectrin antibody, 56A, showing the absence of non-specific labelling. H. The same cells as in G, stained with Hoechst dye to reveal the position of their nuclei. In this and the subsequent figures: ×725; bar, 15 μm, unless otherwise stated.

Fig. 4. Independent capping of thymocyte surface glycoproteins. A, C. Cells demonstrating a capped distribution of the L-C antigen after incubation at 37°C with NDS58. B. The same cells as in A, showing the uncapped distribution of the LSGP revealed by W3/13 staining in the presence of complete capping of the L-C antigen. D. The same cells as in C, showing the uncapped distribution of Thy-1 revealed by OX-7 staining. E. Cells showing a capped distribution of the LSGP after incubation at 37°C with W3/13. F. The same cells as in E, showing the uncapped distribution of the L-C antigen revealed by NDS58 staining in the presence of complete capping of LSGP. G. Cells showing the normal partially capped distribution of Thy-1 after incubation at 37°C with OX-7. H. The same cells as in G, showing the uncapped distribution of the L-C antigen revealed by NDS58 staining in the presence of Thy-1 caps.

Fig. 5. Concurrent partial capping of the L-C antigen and Thy-1. Cells were labelled simultaneously with NDS58 and OX-7 and the appropriate second layers, and incubated at 37°C. A. Partial capping of the L-C antigen. B. Concurrent partial capping of Thy-1 on the same cells as in A. Had the OX-7 anti-Thy-1 monoclonal been omitted, the capping of the L-C antigen would have been complete, resembling that shown in Fig. 3B.

Fig. 6. Sequential capping of thymocyte glycoproteins. A–D. Cells were labelled with W3/13, and the LSGP capped. The cells were then labelled with NDS58 at 0°C, and the L-C antigen induced to cap by incubation at 37°C. A. Cells exhibiting LSGP caps. B. The partially capped distribution of the labelled L-C antigen on the same cells as in A after 5 min incubation at 37°C. C. Cells exhibiting LSGP caps. D. The fully capped distribution of the L-C antigen on the same cells as in C after 30 min incubation at 37°C. E, F. Cells were labelled with NDS58, and the L-C antigen capped. The cells were then incubated with OX-7, and the Thy-1 antigen induced to cap by incubation at 37°C. E. Cells exhibiting L-C antigen caps. F. Partial capping of Thy-1 on the same cells as in E.

Fig. 7. Fodrin and actin distribution in capped thymocytes. Cells were labelled with W3/13 and the LSGP was capped by incubation at 37°C. The cells were then fixed and permeabilized with 0.2% Triton X-100 to permit labelling of the cytoskeletal proteins. A. LSGP demonstrating a capped distribution. B. 56A anti-fodrin labelling of the same cells as A, showing partial co-capping of fodrin. Identical fodrin distribution results were obtained after capping either the L-C antigen or Thy-1. C. LSGP demonstrating a capped distribution. D. TRITC-phalloidin staining for 10 min of the same cells as in C, showing the partial co-capping of F-actin.
Fig. 7. Microtubule distribution in uncapped and capped thymocytes. A. Cells incubated for 60 min at 37°C and then fixed at 37°C, and subsequently permeabilized and labelled with anti-tubulin antibody and a TRITC-conjugated second layer, showing a microtubule network. B. Cells incubated for 60 min at 0°C and then fixed at 0°C, and subsequently labelled as in A. Microtubules are absent, the anti-tubulin fluorescence being uniformly distributed throughout the cytoplasm. C. Cells labelled with W3/13 at 0°C for 2 h and then incubated at 37°C for 20 min, exhibiting LSGP caps. D. The same cells as in C fixed, permeabilized and labelled with anti-tubulin antibody, showing repolymerization of a microtubule network. ×975; bar, 10 μm.

Fig. 8. Effect of microtubule disruption on sequential capping. A,B. Cells were labelled with W3/13, and the LSGP induced to cap at 37°C. Following this, microtubules were depolymerized by cold and 10^-4 M-colchicine was added to prevent repolymerization, the cells were then labelled with NDS58 and the L-C antigen induced to cap at 37°C in the continued presence of colchicine. A. Cells demonstrating LSGP caps. B. The initial stage of capping of the L-C antigen on the same cells as in A after 5 min at 37°C. ×725; bar, 15 μm. C,D. Cells were treated as in A and B, but the colchicine incubations were performed in the absence of L-C antigen labelling. Following a second incubation at 37°C for 30 min, the cells were fixed, permeabilized and stained with anti-tubulin antibody. C. Cells demonstrating LSGP caps. D. The same cells as in C stained with anti-tubulin. No microtubules are visible. ×975; bar, 10 μm.

To study the importance of microtubules during sequential capping, the effect of colchicine disruption of the microtubule network prior to the capping of the L-C antigen was investigated on cells already possessing LSGP caps. After 5 min incubation at 37°C, the L-C antigen had started capping towards the LSGP caps (Fig. 8A,B), and by 20 min more than 90% of cells demonstrated coincident LSGP and L-C antigen caps, as also occurred in the absence of colchicine (Table 1). In a parallel control study, LSGP-capped cells were subjected to identical colchicine treatment, but without the secondary L-C antigen labelling, although the incubations at 0°C and 37°C were still performed. These cells were subsequently fixed and labelled with anti-tubulin antibody, which revealed the absence of microtubules in these colchicine-treated cells (Fig. 8C,D), the amorphous tubulin staining being concentrated under the caps.

Actin and fodrin involvement in capping

To determine whether the capping of the three different major thymocyte surface glycoproteins brought about similar redistributions of the cytoskeletal proteins fodrin and F-actin, capped and uncapped cells were permeabilized and labelled with a polyclonal anti-human brain fodrin antiserum or with TRITC-phalloidin, which binds to F-actin (see Materials and methods).

Table 1. Effect of colchicine on the capping of the L-C antigen on LSGP pre-capped cells

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Distribution of the L-C antigen (% of total cells)</th>
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<tr>
<td></td>
<td>Patched or diffuse L-C antigen and LSGP caps</td>
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<tr>
<td>No colchicine, 5 min at 37°C</td>
<td>32</td>
</tr>
<tr>
<td>No colchicine, 20 min at 37°C</td>
<td>3</td>
</tr>
<tr>
<td>10^-4 M-colchicine, 5 min at 37°C</td>
<td>36</td>
</tr>
<tr>
<td>10^-4 M-colchicine, 20 min at 37°C</td>
<td>7</td>
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In uncapped cells exhibiting a diffuse distribution of surface glycoproteins, both the fodrin and F-actin were distributed in a uniform manner around the periphery of the cell (not shown). However, in all the cells examined with capped L-C antigen, LSGP or Thy-1, a proportion of the fodrin appeared to be concentrated in a submembranous cap directly beneath the capped surface antigens (Fig. 9A,B). The majority of cells maintained a significant level of staining with the anti-fodrin antibody around the periphery of the rest of the cell, although a few demonstrated complete co-capping of fodrin.

A similar redistribution of F-actin was observed in capped cells if the period of incubation with TRITC-phalloidin was restricted to 10 min (Fig. 9C,D). However, prolonged incubation with TRITC-phalloidin gave much higher levels of F-actin staining, which masked the concentration of F-actin under the glycoprotein cap (results not shown).

**Discussion**

**Antigen capping**

As predicted by Corps et al. (1982), the rates and extent of capping of the three thymocyte glycoproteins were dependent upon the degree of cross-linking of the glycoprotein with antibody, being a function both of the number of molecules of a particular antigen present on the cell surface and the concentration of antibody used. The number of molecules of the L-C antigen (Sunderland et al. 1979), the LSGP (Williams et al. 1977) and Thy-1 (Williams et al. 1976) per cell had been determined indirectly by measuring the number of primary antibody molecules bound at saturation. These studies showed that there were approximately 48 000 anti-L-C antigen, 38 000 anti-LSGP and 600 000 anti-Thy-1 antibody molecules bound per cell. It is likely that the numbers of each antigen may be up to twice these values, since each IgG antibody molecule may bind two molecules of antigen.

Consequently, the observed inability of Thy-1 to cap using a high concentration of second antibody may be attributed to the high surface density of Thy-1 molecules, resulting in such a high degree of cross-linking by antibody that the Thy-1 molecules in the membrane are totally immobilized in a continuous two-dimensional antibody–antigen immunoprecipitate covering the entire cell surface. The conditions employed to permit the capping of Thy-1 were a compromise between using a sufficiently dilute second layer to avoid total immobilization and using sufficient of the fluorochrome-conjugated second layer to enable the cells to be visualized and photographed by conventional fluorescence microscopy.

**Fodrin and F-actin distribution**

Using dual-label immunofluorescence, it was confirmed that both fodrin and F-actin become concentrated beneath caps of the L-C antigen, the LSGP or Thy-1. The published fluorescence micrographs of previous reports appear to show all of the anti-fodrin fluorescence (Levine & Willard, 1983; Nelson et al. 1983; Bourguignon et al. 1985) and all of the actin staining (Gabbiani et al. 1977) to be concentrated beneath the capped surface antigen. This appearance may have been the result of weak or incomplete antibody labelling, which failed to show the less-concentrated uncapped actin filaments. The total redistribution of a cytoskeletal protein seems highly unlikely, especially in the case of actin, which often comprises 5–15% of the total cell protein (Geiger & Singer, 1979).

Using TRITC-phalloidin, we were able to manipulate the level of F-actin staining in thymocytes by varying the period of incubation. A short incubation revealed F-actin staining mainly concentrated beneath the capped antigen, with some staining around the periphery of the remainder of the cell. However, longer incubations revealed a much higher level of staining in the uncapped regions, indicating that the majority of the F-actin was not directly involved in the capping process and that the fixation regime employed had not selectively retained only that F-actin associated with the cap.

The incomplete co-capping of fodrin and actin with the surface glycoproteins is significant when trying to interpret the results from the sequential capping of two surface glycoproteins. If these proteins are actively involved in the capping process, the total capping of fodrin and actin would require a redispersion of these molecules before a second capping event could occur. Alternatively, if only a fraction of these molecules is involved in the initial capping event, as our results suggest, subsequent capping events of other antigens could occur immediately. While evidence for recycling of myosin and actin from concanavalin A receptor caps in Dictyostelium discoideum has been presented (Carboni & Condeelis, 1985), it was also observed that α-actinin, which has similarly been implicated in the capping mechanism (Geiger & Singer, 1979; Hoessli et al. 1980), was not recycled. The recycling of the actin and myosin in Dictyostelium may be associated with the subsequent protrusive and motile events of these amoebae. In non-motile cells, endocytosis of the caps may be required before the cytoskeletal components can be recycled.

**Independent capping of surface antigens**

We have demonstrated that the L-C antigen, the LSGP and Thy-1 cap independently of each other on the rat thymocyte, in general agreement with previous data.
cells, on the basis of its association with the cytoskeletal acting as a protein X (Bourguignon & Singer, 1977). Antigens to the cytoskeleton during capping, thereby obligatory co-capping of the 180K thymocyte L-C homme 1972). In particular, the absence of protein fodrin (Bourguignon 1985). However, we cannot rule out the possibility that the binding ratio of L-C antigen to capped LSGP is so low that its function as a protein X is not noticeable by immunofluorescence, or that there may be a sub-population of the L-C antigen on rat thymocytes, not recognized by the NDS58 monoclonal antibody, which is acting in a manner similar to the T-200 antigen on murine cells.

The failure of Thy-1 to be redistributed during the antibody-induced capping of the L-C antigen is directly contrary to the findings of Bourguignon et al. (1978) when capping T-200, and may either reflect basic differences between the rat and mouse systems used or an idiosyncrasy of the mouse cell line employed, which contains exceptionally high levels of T-200, since in the same report it was shown that a mutant of that cell line did not demonstrate co-capping of Thy-1 with T-200.

Sequential and concurrent capping
When capping was induced by warming, following the labelling of two antigens simultaneously, these antigens were always found to cap to the same place on the cell surface, and at the same rate, as has been demonstrated for surface immunoglobulin and HLA antigens on human lymphoid cells (Preud'homme et al. 1972). This could be interpreted in two ways. Either one of the antigens was actively capped and the other antigen, because of the extensive antibody cross-linking causing steric entrapment, was passively dragged with it, or the cross-linking of both antigens was sufficient for them both to become independently attached to the capping machinery and thus, following incubation at 37°C, the two proteins were actively and synchronously capped. The second alternative requires there to be a single polarity for the mechanism responsible for the cap formation, otherwise cells exhibiting two separate caps, one for each antigen, might be expected. The observed co-capping of the two antigens at the same rate is at variance with their capping rates individually. The rate of capping of the LSGP and the L-C antigen together was closer to that of the LSGP alone than to the slower rate observed for the L-C antigen alone. As discussed earlier, the degree of cross-linking is an important factor in determining the rate of capping (Corps et al.

Sequential capping and the cytoskeleton
The role of microtubules and microfilaments in sequential capping
The microtubule network observed in rat thymocytes maintained at 37°C was found to be similar to that in resting murine splenic lymphocytes (Yahara & Kakinoto-Sameshima, 1978; Rogers et al. 1981), with a number of fibres diverging from a single microtubule-organizing centre (MTOC) and ramifying through the narrow layer of cytoplasm surrounding the nucleus in this spherical cell. It is not certain whether the fibres observed represent single microtubules or bundles of several such structures (Yahara & Kakinoto-Sameshima, 1978).

As demonstrated by Tilney & Porter (1967), cold readily disrupted the microtubules, the network observed in lymphocytes being disassembled within 45 min at 4°C (Rogers et al. 1981). Following the capping of the LSGP by incubation at 37°C, the distribution of microtubules was found to be similar to, but less well defined than those of the resting thymocyte, indicating a rapid repolymerization of microtubules on warming, concomitant with cap formation.
There was no evidence of the amorphous co-cap of tubulin reported by Gabbiani et al. (1977) and Yahara & Kakimoto-Sameshima (1978).

The presence of a cellular microtubule network has been found unnecessary for cap formation (Rogers et al. 1981; Edelman et al. 1973; Taylor et al. 1971), but seems to play an important role in positioning the cap in relation to subcellular organelles (Rogers et al. 1981), although the position of the cap in relation to the Golgi apparatus was shown to be different in T and B lymphocytes (Stackpole et al. 1973).

Using cold treatment and colchicine to disrupt and prevent the reassembly of the microtubule network, following the initial capping of the LSGP, we have been able to demonstrate that the subsequent capping of the L-C antigen is not prevented by the absence of microtubules, and that the L-C antigen cap still forms at the site of the LSGP cap. This suggests that while the microtubules may indeed play an important role in orienting the initial cap over a particular region of the cytoplasm, they do not appear to be required to maintain this polarity, which defines the direction in which a second capped antigen is seen to migrate. The cause of the observed partial co-capping of depolymerized tubulin under these conditions is not understood.

The organization of the actomyosin filaments underlying the plasma membrane, which have been proposed to be responsible for capping (Bourguignon & Singer, 1977), may be an important factor in the observed polarity. On the evidence of the data presented in this paper, the involvement of membrane flow (Bretscher, 1976, 1984) in producing the capping polarity cannot be excluded, although the increased insolubility of the L-C antigen and the LSGP to Triton X-100, following antibody cross-linking (Turner & Shotton, 1987, and unpublished data), supports a direct involvement of the cytoskeleton.

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