Phosphorylation of Myosin Light Chain during Capping of Mouse T-Lymphoma Cells

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ABSTRACT Colchicine induces the clustering of at least three different T-lymphoma surface antigens (T200, Thy-1, and gp 69/71) into a cap structure in the absence of any external ligand. In addition, colchicine induces the intracellular accumulation of actin and myosin directly beneath the surface cap structure. We have discovered that myosin molecules (both heavy and light chains) are closely associated with the plasma membrane of T-lymphoma cells. Most importantly, we have found that the 20,000-dalton light chain of lymphocyte myosin is both phosphorylated and preferentially accumulated in the plasma membrane of colchicine-induced capped cells. It is proposed that myosin light chain is directly involved in the activation of membrane-associated actomyosin required for the collection of surface proteins into a cap structure (analogous to muscle cell sliding filament contraction).

Redistribution of lymphocyte surface receptors can be induced either with or without the addition of external ligands (15, 18, 23, 29, 30, 37, 41, 46-48). During the process of receptor movement, surface components first form small clusters (patches), which then aggregate at one pole of the cell into so-called caps. In the case of ligand-dependent capping, the ligand and its receptors appear to be clustered by a cross-linking event at the cell surface that is highly dependent on the valency of the ligand (37). For example, divalent antibodies against surface immunoglobulin (Ig) are required for the induction of Ig capping; monovalent anti-Ig is unable to cause any redistribution of the Ig receptor (30). In addition, capping induced by the lectin, concanavalin A (Con A), requires the tetrameric form of Con A (at the proper concentration) since the dimer form (e.g., succinyl Con A) is not active in cap formation (15, 18). Information concerning ligand-independent capping is very limited. Recently, it has been found that hypertonic media, mitogens, trypsin, and cholinergic drugs are each able to induce surface receptors to form cap structures in the absence of any externally added ligand (29, 46-48). The molecular mechanisms underlying both ligand-dependent and ligand-independent capping are not yet understood.

Previously, we have used a double immunofluorescence labeling technique to simultaneously determine the distribution of certain surface receptors and intracellular actin and myosin in human skin fibroblasts grown in monolayers (9) or in murine lymphoid cells grown in suspension cultures (7, 10, 11). The results of these studies indicate that increased concentrations of actin and myosin are always found directly under the capped membrane (7, 9–11). Consequently, we have proposed that a "transmembrane interaction" (10) occurs between the surface receptors and intracellular actin- and myosin-containing structures that allows the receptors to be actively collected into a cap by a mechanism analogous to the muscle sliding filament contraction process. However, conclusive biochemical evidence for or against these proposed interactions between contractile elements and surface membrane proteins has not been obtained as yet.

In this study, we have focused on the phenomenon of ligand-independent capping in mouse T-lymphoma cells, using both immunocytochemical and immunobiochemical techniques to explore the possible molecular mechanisms involved in the redistribution process. We have established that colchicine induces all three surface proteins tested to form caplike structures in the absence of any external ligand. As with ligand-dependent capping, colchicine-induced capping is sensitive to both temperature and cytochalasin D and is accompanied by the accumulation of intracellular actin and myosin under the surface cap structure. Most importantly, we have found that during colchicine-induced capping the 20,000-dalton light chain of lymphocyte myosin is preferentially accumulated in plasma membrane of T-lymphoma cells and is also phosphorylated. These alterations in myosin light chain phosphorylation suggest that activation of membrane-associated actomyosin (possibly analogous to
that occurring in muscle sliding filament contraction) is required for the collection of surface receptors into a cap structure.

MATERIALS AND METHODS

The mouse T-lymphoma cell line, AKR/J lymphoma line BW 5147 (gift from Dr. R. Hyman, The Salk Institute) grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-activated horse serum (Gibco Laboratories, Grand Island, N.Y.) at 37°C in 5% CO₂/95% air. These T-lymphoma cells display the surface antigens T200, Thy-1, and gp 69/71 (8). Plasma membrane preparations and there are no obvious differences between the two gel banding patterns (Fig. 3A and 3B).

RESULTS

We have explored the effect of colchicine on the distribution of lymphocyte receptors in the absence of added ligand. As shown in Fig. 1a, c, and d, we have found that colchicine alone (i.e., without any ligand) induces a number of lymphocyte surface molecules (e.g., T200, Thy-1, and gp 69/71) to form typical cap structures. In addition, this colchicine-induced capping is dependent on temperature and sensitive to cytochalasin D (Fig. 1b and Table I), as is ligand-induced capping.

We have also utilized the double immunofluorescence labeling technique to simultaneously localize intracellular actin, myosin, and surface receptor molecules on the same cells after colchicine treatment. Our data show that there is a large accumulation of actin molecules at the pole of the cells where surface proteins such as T200, Thy-1, or gp 69/71 are collected into cap structures (Fig. 2a and b). In addition, myosin is aggregated in the same region as actin under the capped structures of the cells after treatment with colchicine (Fig. 2c, d, e, and f).

In this study, the total plasma membrane isolated from untreated cells was compared with that obtained from cells treated with colchicine. In both cases, the plasma membrane was purified at least 20-fold (26) and shown by electron microscopy to contain essentially no other membranous contaminants (e.g., rough endoplasmic reticulum and no detectable nuclear or mitochondrial membranes [data not shown]). Using exponential acrylamide gradient gel electrophoresis to display the membrane polypeptides, we have found that there are at least 30 major polypeptides present in both plasma membrane preparations and there are no obvious differences between the two gel banding patterns (Fig. 3A and B). This latter result is not surprising since capping primarily involves a local rearrangement of surface receptor molecules.

We have noted from the gel patterns that a band traveling
with the same electrophoretic mobility as actin appears to be present in equal amounts in the plasma membrane fraction from both capped and uncapped cells (Fig. 3A and B). With myosin, however, we were not able to determine whether there were any differences on the two SDS polyacrylamide gels because myosin is present in very low amounts in the plasma membrane fraction and comigration of relatively large amounts of membrane polypeptides with molecular weights close to that of myosin could easily obscure both quantitative and qualitative differences.

TABLE I

| Conditions | Drug                          | Capping% |
|------------|-------------------------------|----------|
| 0°C, 60 min| Colchicine (1 X 10^{-4} M)    | 1        |
| 37°C, 15 min| Colchicine (1 X 10^{-4} M)    | 40       |
| 37°C, 15 min| Colchicine (1 X 10^{-4} M) +  | 1        |
|            | cytochalasin D (20 μg/ml)    |          |

* The data are essentially identical for T200, Thy-1, and gp 69/71 capping. Standard deviation is ± 5%.
**Figure 3** SDS polyacrylamide gel analysis of the plasma membranes and myosin-specific immunoprecipitate polypeptides. (A) Plasma membranes from untreated cells. (B) Plasma membrane from colchicine-treated capped cells (arrowhead indicates actin bands). (C) Antimyosin immunoprecipitate from plasma membrane fraction of untreated cells. (D) Antimyosin immunoprecipitate from plasma membrane fraction of colchicine-treated capped cells. (E) A typical gel pattern of following sample: (1) Antimyosin-free (preabsorbed reagent)/(nonspecific) immunoprecipitates from whole-cell homogenate of mouse T-lymphoma as well as plasma membrane fractions of colchicine-treated or untreated cells; (2) antimyosin immunoprecipitates from human skeletal muscle tropomyosin. (F) Human skeletal muscle tropomyosin. (G) Antimyosin immunoprecipitates of whole-cell homogenate of untreated T-lymphoma cells. (H) Antimyosin immunoprecipitates of purified human smooth muscle myosin protein. (Markers used in the study are [in daltons]: myosin, 200,000; β-galactosidase, 130,000; lactoperoxidase, 78,000; bovine serum albumin, 68,000; actin, 40,000; concanavalin A, 25,000; soybean trypsin inhibitor, 17,000; cytochrome c, 12,000.

Consequently, the technique of immunoprecipitation was employed to further study the myosin found in the capped and uncapped cells. The myosin antibodies (rabbit antibodies against human uterus smooth myosin) used throughout these studies were shown to specifically immunoprecipitate only the myosin heavy chain at 200,000 daltons (MH200K) and one of the myosin chains at 20,000 daltons (MLC20K) from smooth muscle systems (Fig. 3H and Fig. 4) (32). Our antimyosin reagent has also been found by immunofluorescence techniques to exhibit a very wide range of cross-reactivity with myosin molecules from a number of different types of animal cells (7, 9–11).

We have carried out immunoprecipitation using the antimyosin antiserum on the whole-cell homogenate fraction from mouse T-lymphoma cells. The proteins in the precipitates were then analyzed by SDS PAGE. Our results show that, in addition to the two typical myosin chains (MH200K and MLC20K), there is an additional polypeptide (28,000 daltons [28K]) present in the immunoprecipitates from both colchicine-treated and untreated cells (Fig. 3G). The presence of the 28K protein could be attributed to either a cross-reactivity of the 28K protein with myosin antibody or to a physical association between the 28K and myosin proteins that exists in vivo. We consider the antimyosin-mediated precipitation to be specific for the following reasons: (a) after the removal of antimyosin, no myosin or little 28K protein can be detected in the nonspecific immunoprecipitates (Fig. 3E); and (b) this reagent does not cross-react with other contractile proteins such as tropomyosin and actin (Fig. 3E, F, and G).

Starting with equal amounts of capped and uncapped cells, we have found that there is relatively little myosin light chain present in the plasma membrane fraction of untreated (uncapped) cells (Fig. 3C). However, MLC20K is readily detected in the immunoprecipitates of the plasma membrane preparation from capped (i.e., colchicine-treated) cells (Fig. 3D). Therefore, during colchicine-induced lymphocyte capping, there appears to be an additional, specific association of MLC20K with the plasma membrane.

Since several studies have suggested that phosphorylation of myosin light chain may be crucial for the overall regulation of contractility in both muscle and nonmuscle cells (1, 2, 12, 13, 16, 31, 33, 49), we decided to determine whether myosin phosphorylation also occurs during colchicine-induced capping. Initially, we metabolically labeled uncapped cells with carrier-free H32PO4 in phosphate-free Dulbecco's modified Eagle's

**Figure 4** Immunoprecipitation of human smooth muscle myosin. Human smooth muscle myosin was isolated (32), iodinated (17, 19), and processed through immunoprecipitation reactions using either antimyosin antibody (A) or antimyosin-free (preabsorbed) antibody (V) reagent according to the procedures described in the text. Immunoprecipitates were analyzed on an exponential polyacrylamide gel gradient (7.0–17.5%) and the discontinuous buffer system described by Laemmli (21). The gels were stained with Coomassie Brilliant Blue, sliced, and counted for their radioactivity (myosin, myosin heavy chain; myosinL, myosin light chain; IgG, immunoglobulin heavy chain; and IgGL, immunoglobulin light chain). Molecular weight markers used in this experiment are identical to those used in Fig. 3.

| Conditions          | 32P/MH200K | 32P/MLC20K | 32P/28K protein |
|---------------------|------------|------------|-----------------|
| Untreated cells     | ND*        | 2.23 × 10⁶ | 3.0 × 10⁶       |
| Colchicine-treated  | ND*        | 1.92 × 10⁶ | 1.5 × 10⁶       |

* ND, not detectable.
medium and determined that both the MLC\textsubscript{20K} and the 28K proteins are phosphorylated but the M\textsubscript{H20K} is not phosphorylated at all (Table II). We then examined myosin and 28K protein from cells induced to form caps by treatment with colchicine and found a very marked increase (at least five- to eightfold) in the phosphorylation of both MLC\textsubscript{20K} and the 28K protein as early as 5 min after incubation of cells with this drug. Again, there was no detectable phosphorylation of M\textsubscript{H20K} (Table II). As a control, we have determined the total amount of radioactive phosphate incorporation into both total cellular proteins and the plasma membrane fraction. Since the incorporation is exactly the same for both untreated and colchicine-treated cells (data not shown), there appears to be no significant difference in the uptake of phosphate by colchicine-treated and untreated cells.

DISCUSSION

Generally, it has been thought that capping requires the binding of a multivalent ligand in order to cross-link the membrane receptors. In this study, we have shown that the antimitotic drug, colchicine, is able to induce the capping of at least three specific mouse T-lymphoma surface receptors (T200, Thy-1, and gp 69/71) in the absence of any external ligand (Fig. 1). Although colchicine has been known to stimulate ligand-dependent capping (e.g., with Con A) (45), the ability of this drug to induce ligand-independent capping in lymphocytes has not been previously reported. Recently, Walter et al. (42) have shown that colchicine is able to induce macrophages to accumulate phagocytic and pinocytic vesicles as well as Con A receptors into a cap structure at one pole of the cell.

The primary mode of action of colchicine has been commonly considered to involve the disassembly of microtubules (4, 5, 22, 27, 44). However, colchicine has also been found to affect several other cellular activities (e.g., nucleoside transport and membrane components) (25, 34, 35). We have obtained preliminary data using lumicolchicine, an analogue of colchicine known to have no effect on microtubule structure, which indicates that it also can induce lymphocyte capping (Bourguignon and Hsing, submitted for publication). Therefore, the involvement of microtubules in the capping process is still an open question. However, the participation of actomyosin molecules in the receptor redistribution of surface receptors is quite definite as shown by both actin and myosin molecules being preferentially accumulated directly underneath the colchicine-treated receptor cap structure (Fig. 2).

By employing an immunoprecipitation procedure specific for myosin, we have determined that our antiamyosin reagent exhibits cross-reactivity with both heavy chain and light chain myosin in purified myosin preparations and whole-cell homogenates of T-lymphoma cells (Fig. 3 G and H, and Fig. 4). The fact that the antiamyosin reagent can also immunoprecipitate a 28K protein together with myosin raises the following two interesting possibilities: (a) the 28K protein may be closely associated with myosin, especially in whole cell and plasma membrane fractions, but is apparently dissociated from myosin during purification; or (b) the 28K protein may contain a myosinlike structure that is immunologically indistinguishable from myosin. Of course, it is also possible that the 28K protein may be somehow induced to form an immunoprecipitate complex with myosin during the immunoreaction. Recently, in an independent study using a nonimmunological approach, we obtained evidence indicating that the 28K protein is a phosphorylated membrane protein that coexists with the MLC\textsubscript{20K} in the lymphocyte capped structure (6). Therefore, we believe that the coexistence of the 28K protein with myosin in the immunoprecipitates most likely results from a real association and not an artificially induced complex. The fact that the protein can be readily phosphorylated and that the extent of its phosphorylation correlates with the capping process suggests that the 28K protein may take part either in the linkage process between plasma membrane and contractile proteins or in the regulatory events involving the actomyosin machinery during lymphocyte capping.

Furthermore, we have found that there is significantly more MLC\textsubscript{20K} protein (~20-fold) in the immunoprecipitate obtained from the plasma membrane of capped cells than in that from the uncapped cells (Fig. 3 C and D). There are two possible reasons for this difference—either there is much less MLC\textsubscript{20K} protein attached (directly or indirectly) to plasma membrane of uncapped cells or the antigenic sites for the myosin antibody are somehow masked in the plasma membrane of the uncapped cell. At this time, we are not able to confirm either possibility. However, in either case, it is clear that colchicine-mediated capping is causing some change related to MLC\textsubscript{20K} that is potentially important for understanding the mechanism of surface receptor movement.

Another potentially important finding is that colchicine induces a significant increase (approximately eightfold) in the phosphorylation of the MLC\textsubscript{20K} (but not M\textsubscript{H20K}), which may be required for the activation of actomyosin complex during receptor capping. The possible relationship between the association of MLC\textsubscript{20K} with the plasma membrane and the extensive phosphorylation of MLC\textsubscript{20K} upon the treatment with colchicine is currently under investigation. We have obtained preliminary data indicating that similar biochemical changes in MLC\textsubscript{20K} occur during ligand-dependent and ligand-independent lymphocyte receptor redistribution.

Phosphorylation of myosin light chains has been shown to take place in a variety of cell types, including skeletal muscle cells (3, 28, 36), smooth muscle cells (12, 16, 33), and nonmuscle cells (2, 13, 31, 38, 49). For instance, the degree of phosphorylation of MLC\textsubscript{20K} (3, 24, 28, 36) appears to correspond to the extent of posttetanic potentiation of skeletal muscle. In smooth muscle cells and a number of nonmuscle cells (e.g., human platelets, proliferating myoblasts, BHK-21 cells), phosphorylation of light chain myosin has been shown to enhance the actin-activated Mg\textsuperscript{2+}-ATPase of myosin molecules (2, 12, 13, 16, 31, 33, 38, 49). This process may be a central event in the overall regulation of contractility in both muscle cells and nonmuscle cells. Consequently, we propose that the observed colchicine-induced phosphorylation of MLC\textsubscript{20K} and, possibly, of the 28K protein may actually modify the attachment of myosin to the plasma membrane, which subsequently activates an actomyosin sliding filament mechanism to collect certain surface receptors into a cap structure.

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