Increased Assembly of Clathrin Occurs in Response to Mitogenic Activation of Murine Lymphocytes*

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The unassembled (soluble) and assembled (particulate) pools of clathrin in murine lymphocytes have been separated by centrifugation, and specifically quantified by immunoblotting of cellular extracts with an anti-clathrin heavy chain monoclonal antibody. In resting spleen lymphocytes only 25–30% of the total cellular clathrin was found to be present in an assembled form. Upon activation of lymphocytes with B or T cell mitogens (lipopolysaccharide or concanavalin A), the levels of assembled clathrin increased to 60% of the total. These changes in the levels of assembled clathrin were not due to an increase in total cellular clathrin concentration following lymphocyte activation, but rather to changes in the steady state ratio of assembled to un assembled clathrin. The increase in assembled clathrin preceded the expression of transferrin receptors, as measured by the cell surface binding of an anti-transferrin receptor monoclonal antibody, and maximal DNA synthesis, indicating that clathrin assembly occurs early after lymphocyte activation and precedes cell division. Immunofluorescence analysis of activated lymphocytes with an anti-clathrin heavy chain monoclonal antibody, and maximal DNA synthesis, indicating that clathrin assembly occurs early after lymphocyte activation and precedes cell division. Immunofluorescence analysis of activated lymphocytes with an anti-clathrin heavy chain monoclonal antibody revealed a punctate staining pattern characteristic of coated pits and vesicles. Activated B lymphocytes displayed particularly prominent staining in the perinuclear region compared to T cells, suggesting that clathrin assembly may be important for B cell functions such as immunoglobulin synthesis or secretion. These results suggest that in lymphocytes, clathrin assembly is a dynamic process that is triggered by mitogenic stimuli.

The formation of coated pits and vesicles is considered to be an essential step in the internalization of ligands through receptor-mediated endocytosis, and in the process of intracellular transport and protein sorting. The formation of these structures involves the continuous assembly of clathrin and other polypeptide components onto plasma membranes, as well as the continuous disassembly of such components from coated vesicles (reviewed by Brodsky, 1988). These active processes result in the formation of two cellular pools of clathrin, one consisting of soluble triskelions found in cytoplasmic fractions of cells and tissues (Bruder and Widenmann, 1986; Goud et al., 1985; Moore et al., 1987), and another consisting of polymerized triskelions found stably assembled onto cellular membrane fractions. It has been reported that whereas the total amount of clathrin (expressed as the percentage of total cellular protein) is constant among different cell lines, the ratio of assembled to unassembled clathrin varies, being higher in cells that are endocytically or exocytically active (Goud et al., 1985).

One of the most rapid responses of cells to several hormones and growth stimuli is a change in receptor-mediated endocytic activity, resulting in an increased uptake of diverse nutrients and macromolecules. For example, within minutes after stimulation of cells by insulin, insulin-like growth factor I or epidermal growth factor (Davis and Czech, 1986; Davis et al., 1986; Wiley and Kaplan, 1984), a rapid increase in the cell surface concentration of transferrin receptors and a concomitant stimulation of iron uptake is observed. In addition, an increase in the number of coated pits in the plasma membrane of PC-12 pheochromocytoma cells has been shown to occur within minutes of stimulation by nerve growth factor (Connelly et al., 1984). More delayed effects of growth factors on endocytic processes also occur, such as the induction by interleukin 2 of transferrin receptors and iron uptake in mitogenically activated human lymphocytes (Neckers and Cosman, 1983).

The observation that receptor-mediated endocytic processes can be regulated by growth factors made it interesting to examine the possibility that the process of clathrin assembly might be affected in response to these stimuli. Lymphocytes are well suited as a model system in which to examine this possibility, in that resting cells express few if any growth factor receptors, and display a low ratio of assembled to unassembled clathrin (Goud et al., 1985). However, exposure of these cells to antigens or mitogens rapidly triggers the expression of various receptors, such as those for insulin, interleukin 2, and transferrin (Neckers and Cosman, 1983; Helderman and Strom, 1979; Kronke et al., 1985). In this study we analyzed the state of clathrin assembly during the mitogenic induction of cellular proliferation in murine lymphocytes.

EXPERIMENTAL PROCEDURES

Cell Isolation and Culture—Lymphocytes were prepared from the spleens of 4–12-week-old female (BALB/c BYJ mice (Jackson Laboratories, Bar Harbor, ME). Spleens were excised under sterile conditions and gently teased in a medium composed of RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (Hyclone), 10 mM each of L-glutamine, nonessential amino acids, and sodium pyruvate, 25 mM Hepes,1 and 50 μM 2-mercaptoethanol (Roman et al., 1984).

1 The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; EGTa, [ethylenesbis(oxymethylenenitrito)tetraacetic acid; TAPS, 3-tris(hydroxyrnethyl)methyl]aminopropanesulfonic acid; LPS, lipopolysaccharide; ConA, concanavalin A.
Cells were cultured at a density of $2.5 \times 10^6$ lymphocytes/ml in 24-well multiwell plates (Corning) at 37 °C in 5% CO$_2$. Lipopolysaccharide (LPS) or concanavalin A (ConA) (Difco) were added to the cultures at a final concentration of 10 µg/ml. In some experiments, erythrocytes were removed from the spleen cell suspensions by hypotonic shock lysis (Moore and Calkins, 1988). The results of experiments done with erythrocyte-depleted cultures were similar to those obtained when these cells were allowed to remain throughout the incubations, which is consistent with the observation that erythrocytes are practically devoid of clathrin (Goud et al., 1985).

B- and T-lymphocytes were separated by direct panning, using 100-µm polystyrene culture dishes coated with affinity purified goat anti-mouse IgG and soybean agglutinin (Zymed). Non-adherent cells were collected, and B cells were recovered by strongly forcing a stream of medium over the plate surface (Mage et al., 1977). Purified B- and T-lymphocytes were then cultured in the medium described above at a density of $2.5 \times 10^6$ cells/ml with either LPS or ConA at a concentration of 10 µg/ml. Cell viability was routinely 90-90%, as assayed by trypan blue exclusion.

**Cell Fractionation** — The separation of the pools of assembled and unassembled clathrin was performed essentially as described by Goud et al. (1985) with some modifications. Lymphocytes (1-5 X 10^5) were harvested by centrifugation in 15-ml conical tubes, the medium was aspirated, and the cell pellets were lysed by vortexing in 500 µl of a buffer composed of 20 mM MES, pH 6.8, 2.5 mM MgCl$_2$, 2.5 mM EGTA, 1 mM 1-10-phenanthroline, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 10 µM leupeptin, 15 µM antipain, 20 µg/ml aprotinin, 10 µg/ml chymostatin, and 0.2% Triton X-100 at 4 °C. The extracts were then centrifuged at 350,000 x g for 15 min in a Beckman Ti-100 table top ultracentrifuge. The supernatants were collected and used as the source of unassembled clathrin. The cell pellets were resuspended in 500 µl of a solution composed of 20 mM TAPS, pH 9.0, and the protease inhibitors described above, and homogenized by five passes in a 2-ml Potter-Elvehjem tissue grinder. After 20-30 min of incubation on ice, the suspensions were centrifuged and the supernatants used as the source of assembled clathrin. In preliminary experiments, the remaining pellet was dissolved and analyzed for the presence of clathrin by immunoblotting. Virtually no remaining clathrin could be detected in this fraction (data not shown).

**Immunoblotting** — Aliquots of the assembled and unassembled clathrin pools were boiled for 2 min in electrophoresis sample buffer (Laemmli, 1970) at a final concentration of 2% sodium dodecyl sulfate and 10 mM dithiothreitol, separated on 6% polyacrylamide gels, and electrophoretically transferred onto nitrocellulose paper (Towbin et al., 1979). After blocking in 5% bovine serum albumin, the blots were incubated overnight with anti-clathrin heavy chain monoclonal antibody Chc 5.9 (Boehringer Mannheim) at a final concentration of 2.5 µg/ml. The blots were then washed and developed with a polyclonal goat anti-mouse immunoglobulin coupled to alkaline phosphatase. Color development was performed according to the manufacturers instructions. The intensity of the colored bands was quantified using a LKB Ultroscan XL laser densitometer. Standard curves were constructed using purified clathrin from rat liver coated vesicles prepared essentially as described by Campbell et al. (1984).

**Anti-transferrin Receptor IgG Binding** — Anti-mouse transferrin receptor monoclonal antibody was purified by batch extraction of culture supernatants of TIB 219 hybridomas (ATCC), using 40-µm Bakerbond ABx resin (Baker). Lymphocytes were harvested, and resuspended to a final concentration of 5 x 10^6 cells/ml in ice-cold Kreb-Ringer buffer with Heps, pH 7.4, supplemented with 3% bovine serum albumin. Aliquots of the cell suspensions (100 µl) were incubated with 20 µg/ml anti-transferrin receptor monoclonal antibody for 2 h at 5°C. The cells were washed twice by resuspension in 1 ml of ice-cold buffer and centrifugation for 1 min in an Eppendorf microfuge, and incubated with 10 µg/ml of a mouse anti-rat polyclonal antibody (Boehringer Mannheim) for 30 min. After two washes, 0.2 µCi of [3H]-protein A (Du Pont-New England Nuclear) were added, and incubations were continued for 30 min. At this time, cells were transferred to tubes containing filter strips, examined in a Beckman Ti-100 table top ultracentrifuge, and the radioactivity associated to the pellets was quantified by gamma-counting.

**Tritiated Thymidine Incorporation** — Lymphocytes were cultured at a density of $2.5 \times 10^6$ cells/ml in 96-well multiwell dishes. Mitogens were added at a final concentration of 10 µg/ml, and at the indicated times, cultures were washed and harvested. Fifty µl of [methyl-3H]thymidine (2 Ci/mmol, Du Pont-New England Nuclear) was added to each well. After 8 h, cells were harvested onto filter strips and washed with saline, 5% trichloroacetic acid, and absolute methanol. The filter discs were dried and radioactivity associated was measured in a β-scintillation counter.

**Immunofluorescence Microscopy** — Purified B- or T-lymphocytes were cytocentrifuged onto microslides and fixed with acetone for 30 min at -20 °C. The excess acetone was allowed to evaporate at room temperature, and the cells were overlaid with fetal calf serum supplemented with 100 µg/ml of a polyvalent goat anti-mouse immunoglobulin (Zymed) to block nonspecific binding. The slide was washed briefly in phosphate-buffered saline, and the preparation was overlaid with 10 µg/ml of monoclonal antibody Chc 5.9. After 60 min of incubation, the slide was washed three times with phosphate-buffered saline, and incubated with 5 µg/ml of an affinity purified goat anti-mouse polyclonal antibody coupled to fluorescein isothiocyanate (TAGO). After 45 min of incubation at 37 °C, the slides were washed in five changes of phosphate-buffered saline over a 45-min period at room temperature. The preparation was overlaid with a solution of 90% glycerol, 10% Tris-buffered saline, and examined on a Zeiss standard WL microscope equipped with a filter for fluorescein. No specific fluorescence staining was observed in experiments where the anti-clathrin antibody was omitted.

**RESULTS AND DISCUSSION**

Clathrin Assembly in Restyng or Mitogen-stimulated Murine Splenic Lymphocytes — To quantify specifically the amount of clathrin present in lymphocyte extracts, an immunoblotting assay using a monoclonal antibody specific for the heavy chain of clathrin (Chc 5.9, Boehringer Mannheim) was employed. This antibody can detect clathrin heavy chain present in crude cell and tissue extracts from diverse species (Bruder and Widemann, 1986). An immunoblot of rat liver-coated vesicles probed with antibody Chc 5.9 followed by a goat anti-mouse immunoglobulin coupled to alkaline phosphatase is shown in Fig. 1. Intense staining of a polypeptide of approximately 180 kDa, corresponding to the molecular weight of clathrin heavy chain, was detected. Quantification of the intensity of the colored band by laser densitometry revealed...
that this technique was sensitive to small amounts of clathrin, and linear over a broad concentration range.

The pools of assembled and unassembled clathrin from resting lymphocytes or from cells that had been stimulated for 48 h with B cell specific (LPS) or T cell specific (ConA) mitogens were separated by centrifugation and aliquots of the cell extracts were analyzed by immunoblotting. In unstimulated cells, clathrin concentration in the unassembled pool was found to be 2–3-fold higher than in the assembled pool (Fig. 2). Upon stimulation of lymphocytes with mitogens the proportion of clathrin in the assembled pool increased significantly. Analysis of the time course of this effect revealed a significant increase in assembled clathrin by 24 h after exposure to LPS or ConA. By 48 h of incubation the proportion of assembled clathrin increased to approximately 60% of the total. The effect of LPS appeared to slightly precede the effect of ConA, being almost maximal after 24 h.

We investigated whether the changes in the ratio of assembled to unassembled clathrin were accompanied by an increase in the total cellular clathrin concentration. In control cells, the concentration of clathrin was approximately 300 ng/10^6 cells, equivalent to approximately 3 x 10^4 triskelions/cell, similar to that previously determined by enzyme-linked immunosorption assays (Goud et al., 1985; Doxey et al., 1987). No significant differences in clathrin concentration (assembled plus unassembled) were detected between control and activated cells during the first 48 h after stimulation with LPS or ConA. After 72 h of incubation with LPS, but not with ConA, the abundance of cellular clathrin increased to approximately 600 ng/10^6 cells, suggesting that increased synthesis of this protein may be required for one or several B-lymphocyte specific functions. These results suggest that the increase in the levels of assembled clathrin observed upon mitogenic stimulation do not result from the induction of clathrin synthesis, but rather from a change in the steady state ratio of assembled to unassembled clathrin.

**Kinetics of Clathrin Assembly, Thymidine Incorporation, and Transferrin Receptor Expression—**Various growth factors and macromolecules which are internalized through receptor-mediated endocytosis are required for B- and T-lymphocyte proliferation (Iacovle and Melchers, 1978; Mendelsohn et al., 1983). If the observed increase in clathrin assembly is required to mediate this process, it should precede or parallel maximal cell proliferation. Fig. 3, *left panel*, shows that under our conditions, stimulation by ConA of thymidine incorporation into DNA was detectable after 24 h, maximal at 48 h, and decreased thereafter. The mitogenic effect of LPS was slightly weaker, being detectable only after 48 h. A comparison with the data in Fig. 2 indicates that clathrin assembly parallels (in the case of LPS stimulation), the maximal effect of these mitogens on cell proliferation, consistent with the notion that clathrin assembly is triggered at early stages of lymphocyte activation preceding cell division.

The molecular mechanisms involved in mediating the assembly of clathrin onto membranes are not known. However, it has recently been proposed that the cytoplasmic domain of the transferrin receptor may serve as an assembly site for coated pit formation at the plasma membrane (Iacovle et al., 1988). Thus, the induction of transferrin receptors in mitogenically activated lymphocytes might trigger clathrin assembly in these cells. To explore this possibility, we compared the kinetics of cell surface transferrin receptor expression to the kinetics of clathrin assembly in response to LPS or ConA. Fig. 3, *right panel*, shows that specific binding of an anti-transferrin receptor monoclonal antibody (Lesley et al., 1984) to the cell surface of ConA-stimulated cells was observed after 24 h, was maximal at 48 h, and remained high through 72 h of culture. Thus, clathrin assembly closely parallels transferrin receptor expression in ConA-stimulated cells, consistent with the notion that this receptor may be an assembly site for clathrin. Anti-transferrin receptor antibody binding to the cell surface of LPS-stimulated cells was detected after 48 h, but was only 20% of that detected after ConA stimulation (Fig. 3, *right panel*). However, clathrin assembly in response to LPS was almost maximal after 24 h and quantitatively similar to that observed in response to ConA (Fig. 2). This kinetic and quantitative lack of correlation between clathrin assembly and transferrin receptor expression during B-lymphocyte activation suggests that at least in these cells, additional factors besides the cytoplasmic
Clathrin Assembly during Lymphocyte Activation

**Fig. 3.** *Left panel*, kinetic analysis of DNA synthesis during lymphocyte activation. Lymphocytes were obtained from spleens of BALB/c mice and cultured in 96-well microtiter plates at a density of $2.5 \times 10^6$ cells/ml. Mitogens were added at time 0 at a concentration of 10 μg/ml. At the indicated times, cultures were pulsed for 8 h with 1 μCi of [3H]thymidine. At the end of the pulse, cells were harvested onto glass filter paper, and trichloroacetic acid-precipitable radioactivity was counted in a β-scintillation counter. Plotted are the means of triplicate determinations from one experiment. Similar results were obtained in three independent experiments. *Right panel*, kinetic analysis of cell surface transferrin receptor expression during lymphocyte activation. Lymphocytes were obtained from BALB/c spleens and cultured in the absence or presence of LPS or ConA. At the times indicated, cells were harvested and incubated with an anti-transferrin receptor monoclonal antibody followed by a rabbit anti-rat polyclonal antibody and 125I-protein A. The counts/min bound to control cells, which do not express transferrin receptors were of 1661 ± 186 (mean ± S.E.), did not change over 72 h of culture, and were subtracted from the counts/min obtained in mitogen-stimulated cells. Plotted are the means ± S.E. of the results obtained in three independent experiments assayed in triplicate.

*Immunofluorescence Analysis of Clathrin in Purified B- and T-lymphocytes*—To further understand the nature of the changes in clathrin assembly that occur during lymphocyte activation, indirect immunofluorescence microscopy of clathrin was performed. Fig. 4 (*top panels*) shows examples of the immunofluorescence micrographs obtained from isolated B- or T-lymphocytes cultured for 72 h in the presence of LPS or ConA, respectively. In both cell types a punctuate fluorescence pattern extending from the perinuclear region to the cell periphery was detected. This staining pattern is characteristic of clathrin-coated pits and vesicles observed in fibroblasts and other cell types (Willingham et al., 1981; Bruder and Widenmann, 1986). Interestingly, many activated B-cells displayed intense staining in the juxtanuclear region. In contrast to activated lymphocytes, resting B- or T-cells (*Fig. 4, lower panels*) displayed diffuse, weak staining with the anti-clathrin monoclonal antibody.

These results are consistent with the biochemical results shown in *Fig. 2*, which indicate that in resting lymphocytes the majority of the clathrin is in a cytoplasmic unassembled form, which would be difficult to detect by immunofluorescence microscopy. In mitogenically activated cells, clathrin is assembled and concentrated in coated pits or vesicles and is thus more readily detectable by this procedure. The enhanced fluorescence in the juxtanuclear region of activated B-cells possibly corresponds to the Golgi apparatus. Although the function of clathrin in the Golgi is not completely defined (Doxey et al., 1987; Wheland et al., 1982; Orci et al., 1986), it
Clathrin Assembly during Lymphocyte Activation

is possible that in these cells it may be important for antibody synthesis and secretion (Kinnon and Owen, 1983).

In addition to clathrin, coated pits and vesicles are composed of assembly polypeptides (Zaremba and Keen, 1983; Robinson and Pearse, 1986; Ahle et al., 1988) which co-assemble with clathrin in vitro systems (Moore et al., 1987), and promote the formation of clathrin baskets (Zaremba and Keen, 1983). The HA-I assembly polypeptides appear to be associated specifically with clathrin-coated membranes of the Golgi, whereas the HA-II peptides are associated with coated pits and endocytic vesicles (Robinson and Pearse, 1986; Ahle et al., 1988), suggesting that clathrin assembly onto specific membrane structures is directed by these polypeptides. Thus, clathrin assembly in lymphocytes may be due to the expression of these polypeptides, and the differences in the pattern of clathrin assembly between B- and T-lymphocytes might be explained by differences in the levels of expression of the two classes of assembly proteins.

In summary, the results presented in this paper indicate that mitogenic activation of resting lymphocytes is accompanied by an increase in the levels of assembled clathrin. This increase is not due to an increased cellular clathrin concentration, but rather to a change in the ratio of unassembled to assembled clathrin. These data support the notion that the dynamic process, which can be rapidly modulated by physiological stimuli (Salisbury et al., 1980; Takemura et al., 1986), in the resting lymphocyte, rapid recruitment of clathrin from an unassembled pool after mitogenic or antigenic stimulation may enable the cell to rapidly internalize growth factors and macromolecules necessary for proliferation. Further studies in these cells may provide insight into the importance of clathrin assembly and receptor endocytosis in lymphocyte activation, and into the biochemical mechanisms that regulate clathrin assembly in intact cells.

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