ACTIN POLYMERIZATION ACCOMPANIES THY-1-CAPPING ON MOUSE THYMOCYTES

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Received 8 December 1980

1. Introduction

Although the mechanism of antibody-induced receptor capping in lymphocytes is still a debated issue [1], it seems almost certain that actin microfilaments play some role in this process. Cytochalasin B (a microfilament disrupting drug) inhibits cap formation and disperses already formed caps [2]. Immunofluorescence studies have shown actin to accumulate beneath the cap [3,4] and, finally, biochemical analysis has shown both H_2-antigens and surface immunoglobulin to be associated with actin when patched or capped [5,6].

Yet, the source of this cap-associated actin is not known. Conveivably, it could have formed by rearrangement of membrane-associated network-actin, by association of the cap with existing cytoplasmic microfilaments or by de novo assembly. This latter possibility has become an attractive mechanism for a variety of cell functions. Recent reports indicate that non-muscle cells maintain relatively large pools of G-actin [7,8], which can be activated (presumably polymerized) upon request, i.e., upon receiving an appropriate stimulus [9,10]. Here we show that such an activation takes place when Thy-1 antigens cap on the surface of mouse thymocytes.

2. Materials and methods

Ethidium bromide was obtained from British Drug House Ltd. DNase I, Sigma electrophoretically-pure, was used without further purification. Double-stranded DNA was Sigma-type 1, from calf thymus. Pure rabbit skeletal muscle G-actin was a gift from Drs A. Oplatka and J. Borejdo. All other reagents used were obtained from Sigma.

2.1. *Antisera*

Anti-Thy-1.2 was an AKR/J anti-C3H/eb serum prepared as in [11]. Its titer was 1:256. FITC-rabbit antimouse IgG (FITC-RAM-IgG) was purchased from Miles Yeda.

2.2. *Determination of actin state and total actin*

Actin was determined by its inhibition of the enzymatic activity of DNase I on double-stranded DNA. The procedure used was similar, in principle, to that in [12]. However, it was rendered more sensitive by determining the DNA digestion through the fall in the fluorescence of a DNA–ethidium complex [13]. The DNase I assay system contained: double-stranded DNA, 6×10^{-2} mg; ethidium bromide, 3×10^{-9} mol; Tris–HCl (pH 8.0) 7.5×10^{-6} mol; $CaCl_2$, 0.6×10^{-6} mol; $MgCl_2$, 6×10^{-6} mol; in a total volume of 3.0 ml. Upon addition of DNase I, an initial short lag period was observed, followed by a linear fall in the fluorescence (excitation 520 nm, emission 602 nm). This linear decrease was proportional to enzyme concentration over 0.05–0.2 μg. Addition of muscle G-actin to the DNase I resulted in an immediate inhibition of its enzymatic activity, 1.1 mol G-actin inhibiting 1.0 mol DNase I. This inhibition was unaltered when performed in the presence of 1% Triton X-100.

Cell samples (10^7 cells) were taken as required and mixed with an equal volume of Tris–saline (pH 7.4) containing 2% Triton X-100 and 0.2 μg DNase I. From this mixture samples were taken at 1 min and 180 min for assay of DNase I activity. Inhibition after 1 min is referred to as the fraction of the cellular actin in a 'G'-like state. The value obtained after 3 h was taken as that of total DNase I-available actin. In the absence of sodium azide this value coincides with immediate inhibition of cell lysates pretreated with 1.5 M guanidine–HCl as in [12]. Inhibition was calculated from an equivalent sample of DNase I incubated under

similar conditions in the absence of cells. It was converted to actin concentrations via a calibration curve of pure muscle actin with DNase I. Protein concentrations in the cells were determined as in [14], using bovine serum albumin as a standard.

3. Results

Total DNase I-available actin in mouse thymocytes was found to give a value of 4.6% of the total cellular protein (table 1). This value is slightly lower than reported values of other non-muscle cells [15]. In untreated cells the 'G'-like actin fraction represented 73% of the total actin. Upon incubation with anti-Thy-1.2 alone, the total actin available for DNase I inhibition remained essentially the same, while the 'G'-like actin consistently increased slightly. This increase was not significant due to the variability of the observed values. Upon addition of the second antibody (FITC–RAM–IgG), capping ensued upon incubation for 30 min at 37°C. Assay of these fully-capped cells showed a highly significant ($p < 0.001$) decrease in the fraction of the actin leading to immediate DNase I inhibition ('G'-like actin). Some 40% of the 'G'-actin was now found to become incapable of rapid inhibition of the DNase I.

The time-course of the 'G'-actin mobilization was next studied (fig.1). Cells were preincubated with anti-Thy-1.2 for 10 min, and then FITC–RAM–IgG was added and the cells were assayed at different times for the fraction of 'G'-like actin. It can be seen that incubation with FITC–RAM–IgG without anty-Thy-1.2 results in constant values of 'G'-actin (~74% of the total). However, incubation with anti-Thy-1.2 alone results again in a slight increase in the 'G'-like actin. Upon addition of FITC–RAM–IgG, initiation of capping leads to a decrease in the fraction of 'G'-

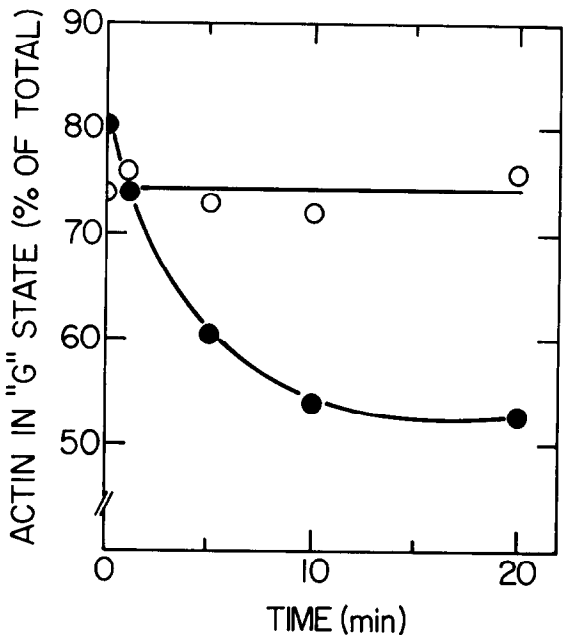

Fig.1. Fraction of thymocyte actin capable of immediate DNase I inhibition during capping of Thy-1.2. Full circles represent capping cells, which have been pretreated with anti-Thy-1.2. (o) Control cells, not pretreated. To both controls and capping cells FITC–RAM–IgG has been added at time = 0. The first two points represent measurements taken just before addition of the second antibody after 10 min preincubation at 37°C.

Table 1
Distribution of 'G'-like actin in capped cells

Additions to cells	'G'-like actin (μg/100 μg protein)	'T' (total) actin (% cell protein)	G/T × 100	n
a. None	3.2 ± 0.7	4.6 ± 1.3	73.2 ± 15	6
b. FITC–RAM–IgG	2.8 ± 0.8	3.5 ± 0.8	. ˜.4 ± 2.6	10
c. Anti-Thy-1.2	4.2 ± 1.9	5.0 ± 2.1	82.3 ± 2.6	2
d. Anti-Thy-1.2 and FITC–RAM–IgG	2.1 ± 0.4	4.8 ± 1.5	46.3 ± 7.0	4

C57BL/6J thymocytes were washed twice in isotonic Tris (pH 7.4) suspended to 2×10^7 cells/ml, mixed 1:1 (v/v) with anti-Thy-1.2 (diluted 1:10) and incubated 30 min at 0°C. After 3 more washes, cells were resuspended to their original volume and preincubated at 37°C for 10 min (c). Then, an equal volume of FITC–RAM–IgG (diluted 1:20) was added and cells incubated for another 30 min (d). Control cells (untreated with anti-Thy-1.2) were similarly incubated for 10 min at 37°C (a) and FITC–RAM–IgG added as before (b)

Table 2
Effect of sodium azide on the state of thymocyte actin

Additions to cells	'G'-actin	'T'-actin	G/T × 100
10^{-2}M azide 1 min at 37°C	2.2	3.5	63.4
10^{-2}M azide 30 min at 37°C	0.9	2.9	34
10^{-2}M azide + anti-Thy-1.2 (1 min)	2.6 ± 0.1	4.2 ± 0.6	63.6 ± 4.3
10^{-2}M azide + anti-Thy-1.2 (30 min)	1.2 ± 0.3	3.1 ± 0.4	37.4 ± 6.2
10^{-2}M azide + anti-Thy-1.2 + FITC–RAM (1 min)	2.7 ± 0.1	4.2 ± 0.5	64.0 ± 8.9
10^{-2}M azide + anti-Thy-1.2 + FITC–RAM (30 min)	1.6 ± 0.3	3.6 ± 0.6	43.7 ± 1.5

like actin readily detected within 5 min and the values fall to a minimum after some 10 min. Under the conditions used for capping, >90% of the cells were found to cap after 30 min.

The next experiments were performed to determine whether the actin mobilization correlates with patching of surface determinants or with their actual capping. The effect of the antibodies was studied in the presence of 10^{-2} M azide which is known to inhibit capping. The results are shown in table 2. The surprising finding was observed that addition of azide alone results in a dramatic decrease in the 'G'-actin. This decrease was already apparent after 1 min becoming much more pronounced after 30 min incubation with the azide (compare with (a), table 1). The addition of the antibodies slightly counteracted the decrease induced by the azide. However, the values at 30 min were lower than those in the absence of sodium azide. It can be also observed in table 2 that in the presence of azide the values of the total actin capable of inhibiting DNase I were lower than those in its absence (table 1).

Attempts to determine total actin by guanidine induced depolymerization [12] gave erratic results which require further study.

4. Discussion

These findings indicate that ~5% of the total mouse thymocyte protein is capable of inhibiting DNase I activity. Of this, ~75% is found in intact cells to be in a 'G'-like form. It should be stressed that 'G'-actin or 'G'-like actin is an operational definition, meaning the fraction capable of immediate DNase I inhibition. Similarly, the 'F'-actin (defined as the difference between the total actin leading to DNase I inhibition and the 'G'-actin) can only tentatively be regarded as filamentous actin. The 'G'-like actin could be truly monomeric actin or some storage form [16, 17], profilactin [18] or some actin gel [19]. Pure 'F'-actin does not immediately inhibit DNase I activity. It is however destabilized by DNase I and depolymerizes to inhibit the DNase I with a half-life of ~5 min [20]. Actomyosin in the absence of ATP is not depolymerized by DNase I and does not inhibit its activity. However, in the presence of ATP actomyosin behaves as 'F'-actin [20].

With these qualifications in mind, it was found that binding of anti-Thy-1.2 and FITC–RAM–IgG causes a depletion of the 'G'-pool by ~40% (presumably leading to actin polymerization). Neither of the antibodies alone has a significant effect on 'G'-actin levels. In this respect, the conditions for polymerization are the same as those for capping. The time-course of polymerization (fig.1), however, seems to precede capping. While it take some 30 min incubation with FITC–RAM–IgG to observe >90% capped cells, the polymerization requires roughly 10 min to reach a plateau.

It is a general, accepted working hypothesis that antibody or ligand-induced receptor capping follows a sequence, which includes:

(i) Ligand-induced receptor microaggregates or patches;
(ii) Association of such clustered receptors with actin microfilaments;
(iii) Active displacement of the clustered receptors towards the uropod, leading to the formation of a cap.

This energy-dependent step involves actomyosin and leads to a redistribution, not only of the clustered receptors but also of the actomyosin which is found mainly under the capped material (see discussion in [21]).

The manner in which clustered receptors become associated with the actomyosin network is not clear. Some models postulate the direct binding of the clusters to preformed actin microfilaments. Alternatively the microfilaments are envisaged to be attached to a component X to which the clusters bind for their ulterior co-capping to the uropod [4]. Neither of the alternative models has received experimental support. A third possibility could be envisaged on the basis of the present data: cluster-formation might be a trigger to lead to a mobilization of precursor actin from some gel or storage form to that of microfilaments. This would mean that actin-microfilaments could actually form beneath a given cluster, might thus become attached to the receptors and be capable of selectively inducing their redistribution.

This hypothesis does not entirely rule out a role for protein X. Conceivably, clustering of receptors forms a nucleation center for actin polymerization. A similar nucleating structure (the acromere) has been described in sperm [22]. Protein X could be an essential component of this nucleation site rather than an anchor for pre-existing filaments.

As mentioned a definite understanding of the azide effect requires further investigation. However, it is apparent that it is a biphasic effect: a rapid depletion of 'G'-actin (presumably by polymerization) followed by transformation of the filaments to a state of partial resistence to depolymerization by DNase I. Since a major consequence of azide incubation is a fall in intracellular ATP this second state may be akin to an actomyosin rigor complex. It is conceivable that myosin binding to actin also induces the observed polymerization. Alternatively, polymerization may be a secondary effect of ATP depletion via changes in the ionic environment. Thus, ATP depletion could lead to Ca^{2+} influx due to an inhibition of the Ca-ATPases of the cell.

Whatever the precise mechanism of azide induced 'G'-actin depletion may be, it suggests an interesting explanation for inhibition of capping. Rather than preventing the pulling of clusters into caps by an energy-dependent actomyosin contraction, inhibition could act at an earlier stage: the azide induced depletion of the 'G'-pool would no longer allow for directional polymerization onto receptor clusters so that the process is inhibited at the stage of selective association of patches with filaments.

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