Elevated expression of the CD4 receptor and cell cycle arrest are induced in Jurkat cells by treatment with the novel cyclic dinucleotide 3′,5′-cyclic diguanylic acid

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Abstract The effect of the novel, naturally occurring nucleotide cyclic diguanylic acid (c-di-GMP) on the lymphoblastoid CD4+ Jurkat cell line was studied. When exposed to 50 μM c-di-GMP, Jurkat cells exhibited a markedly elevated expression of the CD4 receptor of up to 6.3-fold over controls. C-di-GMP also causes blockage of the cell cycle at the S-phase, characterized by increased cellular thymidine uptake, reduction in G2/M-phase cells, increase in S-phase cells and decreased cell division. Additionally c-di-GMP naturally enters these cells and binds irreversibly to the P21^{ras} protein. The effects described appear to be unique for c-di-GMP.

Key words: 3′,5′-Cyclic diguanylic acid; P21^{ras}; CD4 receptor

1. Introduction

3′,5′-Cyclic diguanylic acid (c-di-GMP) (Fig. 1a) is a novel cyclic regulatory nucleotide which has been identified in prokaryotic systems and found to be active also in eukaryotic systems [1]. Our study [2] was the first report on the effects of this nucleotide in a mammalian system: the Molt 4 lymphoblastoid cell line. C-di-GMP was found to dramatically increase cellular thymidine incorporation into Molt 4 cells and, correspondingly, to elevate DNA levels in treated cells but not cell replication. Within the Molt 4 cell, c-di-GMP specifically binds to the p21^{ras} protein, apparently in an irreversible fashion, thereby maintaining it in a fixed active conformation.

The function of Ras in the control of cell fate decision was studied extensively. Ras has been shown to be involved in signaling the differentiation of T-lineage cells and to induce the expression of the T-cell receptors, CD4 and CD8 [3]. Since c-di-GMP activates Ras in Molt 4 cells [2], we sought first to determine whether this unique nucleotide can specifically bind to the p21^{ras} protein in Jurkat cells and secondly whether treatment with c-di-GMP affects the T-cell receptor, CD4 in these cells. For this purpose, we studied the effects of c-di-GMP on this CD4-bearing lymphoblastoid cell line.

In this paper, we report that following its natural entrance into Jurkat cells, c-di-GMP specifically and irreversibly binds to the growth-promoting protein p21^{ras}; that c-di-GMP treatment causes a markedly elevated expression of the CD4 receptor in these cells and that c-di-GMP-treated cells exhibit properties compatible with those of S-phase-arrested cultures. Taken together, we feel that the novel nucleotide c-di-GMP might offer a potentially powerful tool for studying signals involved in T-cell regulation.

2. Materials and methods

2.1. Materials

GTP[S] and affinity absorbent glutardialdehyde-activated beads were purchased from Boehringer, Mannheim, Germany; cyclic GMP, GDP, GMP, p[NH]ppG, GTP, di-isopropyl fluorophosphate (DFP), PMSF, aprotinin, sodium orthovanadate, leupeptin and rabbit anti-rat IgG were from Sigma Chemicals Co. (St. Louis, MO, USA); thin-layer plates of polyethyleneimine (PEI)-cellulose were from Machery Nagel (polygram Cell 300 PEI); rat anti-p21^{ras} monoclonal antibody Y13-259 was from Santa Cruz Biotechnology; rat anti-mouse IgG monoclonal antibody was purchased from Caltag Laboratories (San Francisco, CA, USA). C-di-GMP and [^{32}P]c-di-GMP were synthesized as in [2]. [α-^{32}P]GTP was from Amersham (UK) and [^{3}H]methyl-thymidine from NRC (Negev, Israel).

2.2. Cell lines

Jurkat cells were grown at 37°C in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (Biological Industries, Kibbutz Bet Ha'emek, Israel) as described previously [2].

2.3. [^{3}H]Methyl-thymidine incorporation

Jurkat cells were incubated in 96-well culture plates in the presence of various nucleotides for 24 and 48 h. Cells were then exposed to [^{3}H]thymidine, 1.0 μCi per well, for an additional 4 h. The label content in trichloroacetic acid-precipitable material obtained from the cultures was quantified by liquid scintillation counting.

2.4. Entrance of c-di-GMP to Jurkat cells

Jurkat cells, 2.5×10^{5}, were grown at 37°C in the presence of 50 μM [^{32}P]c-di-GMP (0.5 mCi/μmol) per well in 96-well tissue culture plates. After 24 h, cells were collected by centrifugation, washed and nucleotides extracted as described [2]. Two-dimensional TLC for resolution of nucleotides was done on PEI-cellulose plates, using 1.5 M KH_2PO_4, pH 3.65 (solvent A) for the first dimension and 0.75 M Tris/0.45 M HCl/0.5 M LiCl (solvent B) for the second dimension. Other solvents used were: 0.2 M NH_4HCO_3 (solvent C), 0.2 M $(NH_4)_2CO_3$ (solvent D) and 5.5 M $(NH_4)_2SO_4$, pH 3.5 (solvent E). Labelled compounds were detected by autoradiography and quantified as in [2].

2.5. Immunoprecipitation

Jurkat cells were incubated at 37°C for 24 h in the presence of 5 mM [^{32}P]c-di-GMP (0.8 Ci/μmol) or 10 mM [α-^{32}P]GTP (0.8 Ci/μmol). The cells were removed from the plates, washed three times and lysed as described previously [2]. Post-nuclear supernatants were immunoprecipitated by rotating for 40 min at 4°C using anti-p21^{ras} monoclonal Y13-259 antibody pre-coupled to affinity absorbent glutardialdehyde-activated beads by rabbit anti-rat IgG. All immunoprecipitations were carried out in duplicate and rat anti-mouse IgG monoclonal antibody was used as control. Immunoprecipitates were washed six times with lysis buffer [2] and bound proteins were eluted by boiling in 50 μl of electrophoresis sample buffer for 5 min, followed by SDS-PAGE analysis and autoradiography [2].

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2.6. Enzymatic assay of c-di-GMP

The enzymic assay of c-di-GMP, which is based on the highly c-di-GMP-specific activation of the cellulose synthase of *Acetobacter xylinum*, was employed [4]. The concentration of c-di-GMP in samples was calculated as previously described [5].

2.7. Immunofluorescent staining and flow cytometric analysis

Cells were stained with antibodies following standard procedures. The following antibodies were used: anti-CD4 leu3A and anti-CD8 leu2A (Becton-Dickinson, San Jose, CA, USA) and fluorescing isothiocyanate (FITC) conjugated rabbit anti-mouse IgG (Zymed, San Francisco, CA, USA). DNA content was analyzed by propidium iodide staining of fixed cells following standard procedures. All analyses were carried out using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, USA).

2.8. Immunoblotting

Jurkat cells were lysed in RIPA lysis solution (0.15 mM NaCl/0.05 mM Tris-HCl, pH 7.2/1% Triton X-100/1% sodium deoxycholate/0.1% SDS) at 10^8 cells/ml, and postnuclear supernatants were prepared following standard procedures. Proteins were resolved by SDS/10% PAGE (with 2×10^6 cell equivalents of lysate loaded per lane), transferred onto Immobilon-P membranes (Millipore), and blotted with anti-p56lck rabbit polyclonal antiserum followed by alkaline phosphatase-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates). Color reaction was developed with the BCIP/NBT substrate kit for alkaline phosphatase (Zymed).

3. Results

3.1. Entrance of c-di-GMP into Jurkat cells

To study possible c-di-GMP effects on the T-cell receptor, CD4 in the Jurkat cell line, the entrance of c-di-GMP into the cells was first ascertained. Nucleotides extracted from Jurkat cells, grown in the presence of [^{32}P]c-di-GMP, and then extensively washed, were analyzed for the presence of labelled c-di-GMP by two-dimensional TLC (using solvents A and B) and autoradiography. Fig. 1b shows the autoradiogram obtained, revealing a well-resolved spot which co-migrated with a similarly chromatographed [^{32}P]c-di-GMP marker. Extracts of the spot were subjected to further TLC analysis in various solvent systems. In all cases a single spot exhibiting the distinct mobility of c-di-GMP was detected with R_f values of 0.19, 0.35 and 0.03 in solvents C, D and E, respectively. Similar analysis of the last washing of the cells, as well as extracts of pre-heat-inactivated cells, showed no traces of c-di-GMP. The occurrence of c-di-GMP in the cell extracts described was further established by the ability of extracts derived from unlabelled c-di-GMP-treated cells to activate the c-di-GMP-specific cellulose synthase of *Acetobacter xylinum* [4,5]. These experiments strongly suggest that c-di-GMP enters Jurkat cells. The concentration of free intracellular c-di-GMP was found to reach a value of 35 pmol/10^6 cells, as determined by both TLC and enzymic analysis [5]. This value represents the mean from at least four independent analyses for each method. S.E.M. values were always less than 10% of the mean. Results were statistically significant at $P<0.001$.

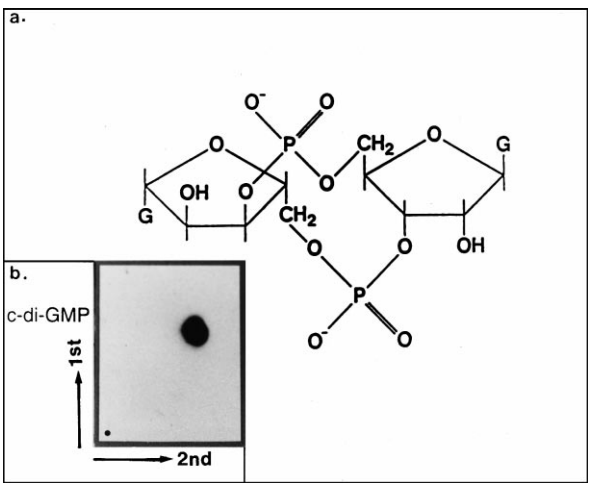

Fig. 1. Structural formula of c-di-GMP and two-dimensional TLC of extracts of [^{32}P]c-di-GMP-grown Jurkat cells. a: Structural formula of c-di-GMP. b: Extracts of Jurkat cells grown in the presence of [^{32}P]c-di-GMP were chromatographed in solvent A in the first dimension and in solvent B in the second dimension, as described in Section 2. Presented is an autoradiogram of the exposed TLC plate. The large dark spot coincides with the position of a similarly chromatographed [^{32}P]c-di-GMP marker.

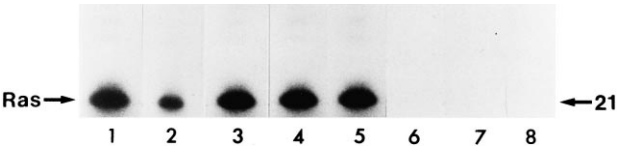

Fig. 2. SDS-PAGE analysis of immunoprecipitates of p21^{ras} from lysates of Jurkat cells labelled with [^{32}P]c-di-GMP or [α-^{32}P]GTP. Jurkat cells were incubated with either 5 nM [^{32}P]c-di-GMP (0.8 Ci/μmol; lanes 1, 3–6, 8) or 10 nM [α-^{32}P]GTP (0.8 Ci/μmol; lanes 2 and 7) in the absence of additional unlabelled nucleotides (lanes 1, 2, 7, 8) or in the presence of either 5 μM c-di-GMP (lane 6), 5 μM GTP (lane 3), 5 μM GDP (lane 4) or 5 μM GMP (lane 5). Cells were lysed and immunoprecipitated as described in Section 2 with the following antibodies: lanes 1–6: Ras-specific monoclonal antibody Y13-259; lanes 7 and 8: rat anti-mouse IgG monoclonal antibody. 10 μM unlabelled c-di-GMP had no effect on [α-^{32}P]GTP labelling.

3.2. C-di-GMP specifically binds to P21^{ras} in Jurkat cells

To determine whether c-di-GMP binds to p21^{ras} in the CD4+ cell line, Jurkat cells previously incubated for 24 h with either [^{32}P]c-di-GMP or [α-^{32}P]GTP were lysed and then immunoprecipitated with the anti-Ras monoclonal antibody Y13-259. The precipitates were subjected to SDS-PAGE analysis and autoradiography. As seen in Fig. 2, lanes 1 and 2, a labelled band of 21 kDa was observed in both immunoprecipitates. Notably, the label intensity of the band derived from c-di-GMP-treated cells is considerably higher than that derived from GTP-treated cells, in spite of the relatively lower molar concentration of the c-di-GMP employed. This band was not seen when Y13-259 was substituted with rat anti-mouse IgG monoclonal antibody (lanes 7 and 8). Labelling by [^{32}P]c-di-GMP was not significantly affected (less than 5% as analyzed by scanning densitometry) in the presence of unlabelled GTP, GDP and GMP at 1000-fold molar excess (lanes 3, 4 and 5). In contrast, unlabelled c-di-GMP, at 1000-fold molar excess, entirely blocked this labelling (lane 6). The results of those competition experiments suggest that c-di-GMP itself binds to p21^{ras}, rather than a guanosine phosphate which may be generated from the cyclic dinucleotide. This conclusion is compatible with the demonstrated presence of c-di-GMP within cells incubated with the dinucleotide. It is noteworthy that unlabelled c-di-GMP at 1000-fold molar excess did not significantly affect the labelling by [α-^{32}P]GTP (less than 5% as analyzed by scanning densitometry) (data not shown). This could imply that c-di-GMP may bind to a

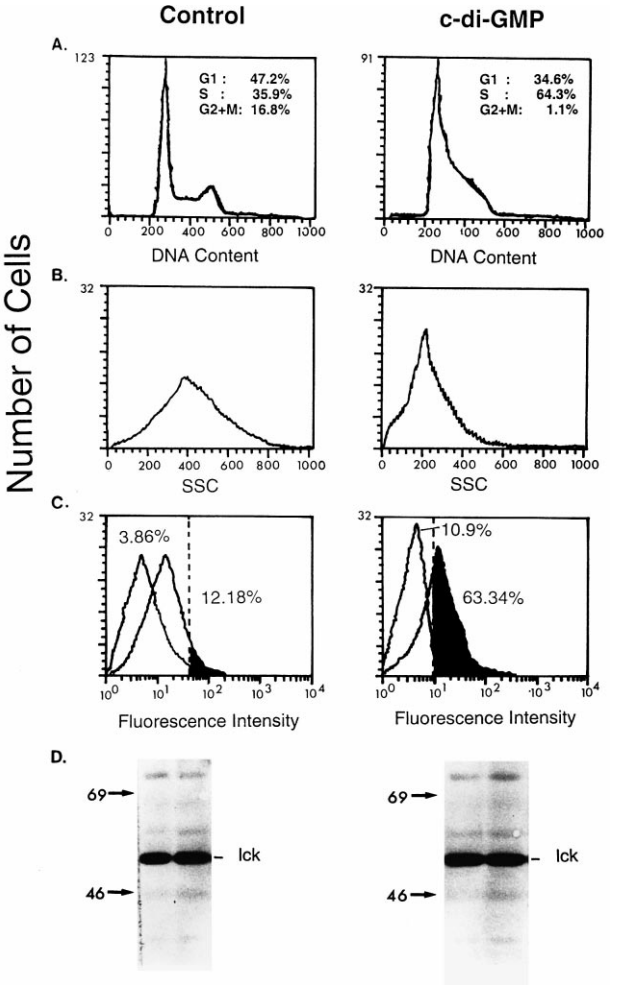

Fig. 3. Effect of c-di-GMP on cell cycle, light scattering behavior and CD4 and lck expression in Jurkat cells. Jurkat cells were untreated (control) or treated with 50 μM c-di-GMP for 24 h (A, B). Cells were fixed with ethanol, stained with propidium iodide and analyzed by flow cytometry. A: Plot of cell number against DNA content. B: Right angle light scatter histogram. C: Cells were harvested and analyzed for CD4 expression by flow cytometry as described in Section 2. The left peaks represent analyses of control cells without first antiserum. The vertical line indicates the position of control staining. The filled area represents CD4-positive cells and values denote percent CD4-positive cells. D: Cells lysates were resolved in SDS-10% PAGE and immunoblotted with an anti-lck antiserum, as described in Section 2. Samples shown are in duplicate. Data presented in A, B, C and D represent one of at least three independent experiments, each performed in duplicate. Results similar to control were obtained when cGMP or GTP were used in the different assays.

non-nucleotide-specific region of $p21^{ras}$, a conclusion compatible with the observed inability of the various guanine nucleotides to inhibit the interaction between c-di-GMP and RAS.

3.3. C-di-GMP specifically elevates CD4 expression in Jurkat cells

The results in Fig. 3C show that when incubated with Jurkat cells c-di-GMP at 50 μM induces a 6.3-fold upregulation of CD4 over control (52.44% CD4-positive cells versus 8.32). The effect was dose-dependent and treatment with 100 μM c-di-GMP resulted in a 9.1-fold increase over control (data not shown). The c-di-GMP-induced upregulation of CD4 was specific and was not observed with c-GMP or GTP, nor with the unhydrolyzable GTP analogues, GTP[S] and p[NH]ppG, at concentrations up to 100 μM. C-di-GMP affected only CD4 expression, but had no effect on the expression of CD8 (data not shown).

3.4. CD4 elevated expression is not accompanied by a commensurate increase in p56lck

Since CD4-derived signals are transduced into the cell by associated tyrosine kinase p56lck molecules [11,12], we sought to find out whether the amount of lck in c-di-GMP-treated cells was correspondingly elevated. For this purpose, 24 h c-di-GMP-treated Jurkat cells lysates were immunoblotted with anti-lck antibody and, as revealed in Fig. 3D, lck levels were not significantly increased over control (less than 4% as quantified by scanning densitometry).

3.5. Effects of c-di-GMP on [^{3}H]thymidine incorporation into Jurkat cells

C-di-GMP has been shown to dramatically increase [^{3}H]thymidine incorporation (up to 200-fold of control) into Molt 4 cells [2]. As seen in Table 1, when incubated for 24 h with Jurkat cells, c-di-GMP at a concentration of 50 μM caused a 43-fold increase in thymidine incorporation into the cells. Fold stimulation over control samples increased further at 100 μM c-di-GMP and following incubations for 48 h. This marked effect was not observed with c-GMP, GMP or GTP (Table 1), nor with the unhydrolyzable guanine nucleotides, GTP[S] and p[NH]ppG, which at concentrations up to 100 μM did not affect [^{3}H]thymidine uptake (results not shown). On the other hand, c-di-GMP had a negative effect on cell replication since the number of treated cells did not increase significantly after 24 and 48 h of incubation, as compared to that observed in control cultures which increased by 44 and 90%, respectively.

3.6. Cell cycle arrest in c-di-GMP-treated cells

Since cells treated with c-di-GMP exhibit a dramatic increase in [^{3}H]thymidine incorporation, we further characterized the state of c-di-GMP-treated cells by flow cytometry (Fig. 3A). When cells were treated with 50 μM c-di-GMP for 24 h, the percentage of cells in S-phase increased by 79%, with almost complete disappearance of G2/M-phase cells which decreased by 93%. These results suggest that c-di-GMP may cause a blockage of the cell cycle at the S-phase. C-di-

Table 1
Effects of c-di-GMP concentration on [^{3}H]thymidine incorporation into Jurkat cells with time

Addition	[^{3}H]-Thymidine incorporation (cpm $\times 10^3$)	
	24 h	48 h
–	1.5	2.4
50 μM c-di-GMP	65	160.8
50 μM c-GMP	2.4	2.4
50 μM GMP	2.2	2.4
50 μM GTP	2.7	2.6
100 μM c-di-GMP	97.5	192

Jurkat cells were incubated for 24 and 48 h in the absence or presence of the various guanine nucleotides, at concentrations of 50 and 100 μM for c-di-GMP and 50 μM for c-GMP and GTP, then pulsed with [^{3}H]thymidine and assayed as described in Section 2. Values represent means from at least three independent experiments, each performed in triplicate. S.E.M. was always less than 10% of the mean. $P < 0.001$.

GMP treatment did not increase the rate of cell death relative to that in control cultures, since the amount of fragmented DNA did not increase in treated cells. This was verified when cell death was evaluated by trypan blue exclusion. Notably, in these experiments too, total cell number in c-di-GMP-treated cultures did not significantly increase as assessed by cell counting. Thus, although c-di-GMP-treated cultures exhibited a high percentage of S-phase cells, overall cell replication was drastically impaired.

Light scattering properties of cells are dependent on cell size. In a randomly proliferating cell population, the larger cells in the G2/M-phase have increased forward and right angle light scatter values compared with their smaller counterparts in G1 phase [6]. Light scatter analyses of c-di-GMP-treated cells revealed a significant shift in population cell size, showing decreased forward (data not shown) and right angle light scatter values compared with controls (Fig. 3B), indicating cells to be of a smaller size. The c-di-GMP effects on cell size and cell type distribution were specific and were not observed in cells treated with c-GMP, GTP[S], p[NH]ppG or GTP at 50 μM for 24 h.

4. Discussion

This study reports on the specific binding of the unique, naturally occurring regulatory nucleotide c-di-GMP to the $P21^{ras}$ protein and its ability to stimulate expression of the CD4 receptor in the CD4+ Jurkat cell line. Ras is known to be involved in signaling the differentiation of T-linkage cells [7,8], and in its activated form has been shown to cause transition of double negative (DN) prothymocytes to double positive (DP) thymocytes, thus inducing the expression of the T-cell receptors CD4 and CD8 [3]. C-di-GMP was demonstrated to freely enter Jurkat cells (Fig. 1b) and was found to bind to the $P21^{ras}$ protein. Since this binding is apparently specific and cannot be blocked by other guanine nucleotides (Fig. 2), and since it remains attached to $P21^{ras}$ after boiling, suggesting that they are covalently linked, it appears that the cyclic nucleotide binds irreversibly to $P21^{ras}$, apparently in the non-nucleotide-specific region of RAS, and possibly maintaining it in a fixed conformation. Since the upregulation of the CD4 receptor observed in c-di-GMP-treated cells appear to be unique to c-di-GMP it is tempting to speculate that the irreversible binding of c-di-GMP with $p21^{ras}$ may result in a complex that possibly maintains the protein in a fixed active form, which in turn may relate to the markedly induced overexpression of CD4 in c-di-GMP-treated cells. Introduction of a constitutively activated Ras expression construct was reported to be sufficient to induce the transition from DN prothymocytes to the DP stage [3], the expression of activated Ras was not sufficient to induce negative selection and maturation towards a single positive form, either CD4 or CD8. In our case, c-di-GMP treatment of single positive Jurkat cells specifically induced CD4 expression (6.3-fold over controls) but did not affect expression of the CD8 receptor. Considering other reports on elevated expression of the CD4 receptor using thymocytes from CD4 transgenic mice [9] and CD4-transfected human T-cells [10], we obtained our effect by exposing Jurkat cells to the novel cyclic nucleotide, and it should be noted that c-di-GMP-treated cells were not genetically altered and the effects described here were observed under conditions which we feel avoid perturbation of cell genetic regulation.

CD4-mediated T-cell activation is trans-signalled through the lymphoid-specific tyrosine kinase p56lck [11]. To assist receptor-mediated antigen recognition, p56lck associates physically and functionally with the CD4 receptor [12]. It would thus be reasonable to expect that increased expression of the CD4 receptor would cause a concomitant elevation in the level of its intracellular signaling counterpart p56lck. However, our experiments show that the excessive upregulation of CD4 in c-di-GMP-treated cells was not accompanied by a commensurate increase in associated p56lck. In this respect our results are in accord with the overexpression of CD4 in mouse T-cells which similarly did not result in a proportionate increase in p56lck molecules [9].

We have previously shown that c-di-GMP exerts an unusual effect on the cell cycle of Molt 4 lymphoblasts, causing them to enter the S-phase uncoordinated with their overall replication rate [2]. Flow cytometric analysis of c-di-GMP-treated Jurkat cells (Fig. 3A) revealed that the percentage of cells in the S-phase increased by 79%. Concomitantly there was a slight fall in the proportion of cells in the G1-phase with an almost complete disappearance of G2/M-phase cells. While the occurrence of a high percentage of c-di-GMP-treated cells in the S-phase is consistent with their elevated uptake of thymidine (Table 1), these effects were, surprisingly, not accompanied by an expected corresponding increase in cell division but rather in cessation of cell proliferation, which taken together strongly suggest that an S-phase arrest is induced in Jurkat cells by c-di-GMP.

Cell size is a critical parameter in the mechanisms which control cellular proliferation in the yeast system [13], and a reduced cell size can be obtained when overexpression of mammalian cyclins, such as D1 and E, occurs [13]. Light scatter analysis of c-di-GMP-treated cells (Fig. 3B) revealed a significant shift in population cell size, indicating cells to be of a smaller size, which is compatible with such cells being mostly in G1- and S-phases where cells are usually of smaller size. Considering these findings and the dramatic increase in cellular thymidine uptake accompanied by blockage of cell proliferation, it appears that although c-di-GMP-treated cells could enter S-phase and augment their DNA content, they could not complete S-phase and undergo mitosis. It is noteworthy that c-di-GMP-treated Jurkat cells were grown in 10% fetal calf serum-containing medium, providing optimal conditions for cell growth and proliferation, thereby avoiding the deleterious effect on cell fragility, viability, growth rate and metabolism, reportedly associated with changes in medium serum levels [14].

It is believed that activated Ras plays an important role in the growth and cell size regulation of mammalian cells. Introducing activated Ras into Schwann cells or rat embryo fibroblasts has been reported to result in arrest of growth [15,16], and inhibition of proliferation was observed when PC12 cells were microinjected with Ras protein [17].It may be suggested that the S-phase arrest apparently induced by c-di-GMP in Jurkat cells could be related to the irreversible binding of c-di-GMP to $P21^{ras}$, which possibly maintains the proto-oncogenic protein in a fixed active state.

Taking all the data presented here into consideration, we feel that our experimental system, using the naturally occurring guanine nucleotide c-di-GMP, might offer a potentially powerful means of studying signals involved in the control of cell fate decision in lymphocytes.

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