The novel cyclic dinucleotide 3'-5' cyclic diguanylic acid binds to p21^{ras} and enhances DNA synthesis but not cell replication in the Molt 4 cell line

Dorit AMIKAM,*† Osnat STEINBERGER,* Tamar SHKOLNIK† and Zvi BEN-ISHAI*

*Molecular Genetics Unit, Rambam Medical Center, and Rappaport Institute of Medical Sciences, Haifa, Israel, and †Clinical and Research Laboratories, Nahariya Regional Hospital, Nahariya, Israel

1. The effect of the novel, naturally occurring nucleotide 3'-5' cyclic diguanylic acid (c-di-GMP) on the lymphoblastoid Molt 4 cell line was studied. When exposed to this guanine nucleotide, Molt 4 cells exhibited a marked increase in [³H]thymidine incorporation, up to 200-fold at 50 μ M c-di-GMP. Correspondingly, the DNA content of the treated cells was 9-fold higher than untreated cells. Stimulation of [³H]thymidine incorporation into the cells was time- and concentration-dependent. This effect was specific and was not observed with GMP or cyclic GMP, nor with the unhydrolysable GTP analogues, guanosine 5'-[γ -thio]triphosphate and guanosine 5'-[β -imido]triphosphate. C-di-GMP entrance into the cells was experimentally verified and occurred without using any means of cell permeabilization. SDS/PAGE analysis of cells exposed to [³²P]c-di-GMP, followed by autoradiography, revealed the labelling of

three low-molecular-mass proteins at 18–27 kDa. The labelling is highly specific to c-di-GMP and its extent was not affected by other guanine nucleotides. 2. One of the c-di-GMP-binding proteins was found to be the p21^{ras} protein, by immunoprecipitation with the anti-Ras monoclonal antibody Y13-259. The effects described appear to be unique for c-di-GMP and, taken together, raise the possibility that an irreversible binding of this guanine nucleotide to the growth-promoting p21^{ras} protein results in a fixed active conformation of this protein affecting DNA synthesis. Strikingly, although at 48 h of growth markedly high DNA levels were found in Molt 4 cells treated with c-di-GMP, this guanine nucleotide had no effect on cell replication during this period. Thus Molt 4 cells exposed to c-di-GMP enter the S phase uncoordinated with their overall replication rate.

INTRODUCTION

Guanine nucleotides and stable GTP analogues, such as guanosine 5'-[γ -thio]triphosphate (GTP[S]) and guanosine 5'-[β -imido]triphosphate (p[NH]ppG), have long been used for studying GTP-binding proteins, including those encoded by members of the p21^{ras} gene family [1–4]. The GTP-bound Ras conformation seems to represent the active, growth-promoting state of the protein, whereas the GDP-bound form appears to be inactive [5,6].

Cyclic nucleotides, such as cyclic AMP and cyclic GMP, have been recognized as mediators of diverse activation processes in different biological systems and have also been implicated in the initiation of the lymphocyte proliferation process. Recently, a novel cyclic nucleotide, 3'-5' cyclic diguanylic acid (c-di-GMP) (Figure 1) has been identified in prokaryotic systems and found to be active also in eukaryotic systems [7]. Its formation from two molecules of GTP is catalysed by diguanylate cyclase and its degradation is catalysed by a c-di-GMP-specific phosphodiesterase. Since guanine nucleotides in general appear to play a role in the regulation of eukaryotic processes, and in view of the unique structure and the natural occurrence of c-di-GMP, we found it interesting to examine whether this nucleotide has any effect on mammalian systems.

In this paper, we describe the natural entrance of c-di-GMP into lymphoblastoid Molt 4 cells without prior permeabilization of the cells. When incubated with these cells, c-di-GMP, unlike other guanine nucleotides, including the non-hydrolysable GTP

analogues, GTP[S] and p[NH]ppG, causes a dramatic increase in cellular [³H]thymidine incorporation. Within the Molt 4 cell, c-di-GMP specifically binds to the growth-promoting protein, p21^{ras}, which may result in a fixed 'active' conformation of the protein. Interestingly, this, in turn, is accompanied by a dramatic elevation of DNA synthesis which is not followed by an increase in cell proliferation. Thus, cells treated with c-di-GMP enter the S phase uncoordinated with their overall replication rate. Our

Figure 1 Structural formula of bis-[3'-5']-cyclic diguanylic acid (c-di-GMP)

system may serve as an attractive model for studying cell-cycle-affecting proteins, such as cyclins, and may be useful in elucidating finer details of the signalling network of the Ras superfamily G-proteins.

MATERIALS AND METHODS

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, U.S.A.; tri-reagent kit was from MRC, Inc., Cincinnati, OH, U.S.A.; 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, Inc.; rat anti-(mouse IgG) monoclonal antibody was purchased from Caltag Laboratories, San Francisco, CA, U.S.A. [α -³²P]GTP (400 Ci/mmol) and [³⁵S]methionine (> 1000 Ci/mmol) were from Amersham International, Amersham, Bucks., U.K. [³H]Methyl thymidine (5 Ci/mmol) was purchased from the Nuclear Research Center, Negev, Israel. C-di-GMP and [³²P]c-di-GMP were synthesized enzymically and purified from GTP or [α -³²P]GTP as described by Ross et al. [8]. The material was > 98% pure as judged by HPLC, TLC and enzymic analysis. Most of the experiments described were repeated with chemically synthesized c-di-GMP [9] with identical results.

Cell lines

Molt 4 lymphoblastoid cell line was grown at 37 °C in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated foetal-calf serum (Biological Industries, Beit Ha'emek, Israel) in a humidified atmosphere of 7% CO₂ in air and subcultured at 72 h intervals.

[³H]Methyl thymidine incorporation

Molt 4 cells (5×10^4) were incubated in 96-well culture plates in the presence of various nucleotides for 4–96 h. Cells were then exposed to [³H]thymidine, 1.0 μ Ci 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.

DNA extraction

Molt 4 cells were cultured for 48 h. Cells were enumerated using a haemocytometer and DNA was extracted using the tri-reagent kit supplied by MRC Inc., according to the manufacturer's instructions. DNA concentrations were determined spectrophotometrically at 260 nm.

Morphological analysis

Cells exposed to [³H]thymidine, as described above, were washed in PBS, pH 7.5, mounted on glass slides, fixed with 4% glutaraldehyde and embedded in epon 812. The slides were coated with nuclear track emulsion (NTB-2, Kodak, Rochester, NY, U.S.A.) and placed in light-tight boxes for 3 weeks at 4 °C. Autoradiographs were developed in D-170 developer (Kodak) at 18 °C, fixed and stained with 1% Toluidine Blue solution, pH 8.0.

[³⁵S]Methionine incorporation

Molt 4 cells were cultured for 36 h and then pulsed for another 12 h with [³⁵S]methionine (0.2 μ Ci per well). Label content in trichloroacetic acid-precipitable material was quantified by liquid scintillation counting.

Entrance of c-di-GMP to Molt 4 cells

Molt 4 cells, 5×10^4 , were grown at 37 °C in the presence of 50 μ M [³²P]c-di-GMP (0.5 mCi/ μ mol) per well in 96-well tissue-culture plates. At various incubation periods, cells were collected by centrifugation and washed three times with ice-cold PBS and then heated at 100 °C for 3 min. Nucleotides were extracted twice with 0.50 ml of 65% (v/v) ice-cold ethanol. The combined extracts were centrifuged and the supernatant used for chromatographic analysis. Two-dimensional TLC for resolution of nucleotides was done as previously described [10] on PEI-cellulose plates (20 cm $\times$ 20 cm), prewashed with 0.5 M LiCl, rinsed with distilled water and dried before use. Solvents used were: 1.5 M KH₂PO₄, 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 in this work were: 0.2 M NH₄HCO₃ (solvent C), 0.2 M (NH₄)₂CO₃ (solvent D) and 5.5 M (NH₄)₂SO₄, pH 3.5 (solvent E). Labelled compounds were detected by autoradiography (exposure time varied from 1 day to 2 weeks). For quantification, the radioactive spots were cut out from the plates and counted in toluene-based scintillation fluid or their intensity determined by densitometric scanning, using a Vilber Lourmat imaging system (Bio Profile V 5.0).

Nucleotide-protein binding

Molt 4 cells were incubated at 37 °C for 48 h in the presence of 5 nM [³²P]c-di-GMP (0.8 Ci/ μ mol) or 10 nM [α -³²P]GTP (0.8 Ci/ μ mol). The cells were removed from the plates and washed three times with cold PBS. DFP was added and the mixture incubated at 37 °C for 15 min, followed by two washings with PBS. Cells were lysed by shaking in 1 ml of ice-cold lysis buffer containing 20 mM Tris, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 10 mM EDTA and 100 mM NaF, to which 1 mM PMSF, 0.02 M aprotinin, 0.02 M sodium orthovanadate and 10 μ g/ml leupeptin were added. Nuclei were removed by centrifugation at 15000 *g* at 4 °C for 15 min. To the post-nuclear supernatants were then added 50 μ l of electrophoresis sample buffer, the mixture was heated at 100 °C for 5 min and then resolved by SDS/PAGE with 12% acrylamide gels [11]. The gels were stained with Coomassie Brilliant Blue, destained, dried and autoradiographed. The intensity of radio-labelled bands was determined by densitometric scanning.

Immunoprecipitation

Cells were cultured, incubated with [³²P]c-di-GMP or [α -³²P]GTP and lysed as described above. 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 and bound proteins were eluted by boiling in 50 μ l of electrophoresis sample buffer for 5 min, followed by SDS/PAGE analysis and autoradiography.

Enzymic 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 [12,13]. The concentration of c-di-GMP in the sample was calculated by comparing the increase in cellulose synthase activity above basal activity observed in its presence with corresponding increases in synthase activity brought about by the addition of various concentrations of c-di-GMP, as described [10].

RESULTS

Effects of c-di-GMP on [³H]thymidine incorporation into the Molt 4 cell line

At a concentration range of up to 50 μ M, c-di-GMP, unlike its lower homologue cyclic GMP, exerted a marked increase of up

Figure 2 (a) Effect of c-di-GMP concentration on [³H]thymidine incorporation into Molt 4 cells and (b) time course of thymidine incorporation in the absence or presence of c-di-GMP

(a) Molt 4 cells were incubated for 48 h in the presence of c-di-GMP (●), cyclic GMP (▲) or GMP (○) over a concentration range of 1–100 μ M and then pulsed with [³H]thymidine and assayed as described in the Materials and methods section. Label uptake in control cultures was 1500 c.p.m. Values represent means from at least three independent experiments, each performed in triplicate. S.E.M. was always less than 10% of the mean. $P < 0.001$. (b) Molt 4 cells were incubated for the times indicated in the absence (○) and presence of either 50 μ M c-di-GMP (●) or 50 μ M cyclic GMP (▲) and then pulsed with [³H]thymidine and assayed as described in the Materials and methods section. Values represent means from at least three independent experiments, each performed in triplicate. S.E.M. was always less than 10% of the mean. $P < 0.001$.

Figure 3 The effect of c-di-GMP on Molt 4 cells replication

Molt 4 cells were incubated for the times indicated in the absence (○) and presence (●) of either 50 μ M c-di-GMP or 50 μ M cyclic GMP (△) and then enumerated using a haemocytometer as described in the Materials and methods section. Values represent means from at least three independent experiments, each performed in triplicate. S.E.M. was always less than 10% of the mean. $P < 0.001$.

to 200-fold on [³H]thymidine incorporation into Molt 4 cells. At a higher concentration range, [³H]thymidine incorporation reached a steady state. This dramatic effect was not observed with cyclic GMP or GMP (Figure 2a), nor with the unhydrolysable guanine nucleotides, GTP[S] or p[NH]ppG (results not shown) which, at all concentrations tested, did not affect the uptake of [³H]thymidine. Stimulation of [³H]thymidine incorporation into the cells varied with the time of exposure to c-di-GMP, and fold stimulation over control samples was maximal following incubation for 48 h (Figure 2b). A similar stimulation of [³H]thymidine incorporation into c-di-GMP-treated cells was also observed when employing serum-free medium (results not shown).

In order to assess the DNA content of c-di-GMP-treated cells compared with control cells, DNA was extracted from a counted number of cells exposed to c-di-GMP for 48 h, measured and found to be 9-fold higher than in untreated cells (81.9 versus 9.1 μ g of DNA per 10^6 cells). Acid-precipitable specific radioactivity of the [³H]thymidine-labelled DNA from treated cells was 22.3-fold higher than that of untreated cells or for cells treated with cyclic GMP or GMP (37×10^3 versus 1.7×10^3 c.p.m./ μ g of DNA). The values of both of these assays represent means from at least three independent experiments, each performed in duplicate. S.E.M. values were always less than 10% of the mean. Results were statistically significant at $P < 0.001$.

Notably, up to 48 h, c-di-GMP had no effect on cell replication and the number of treated cells varied with time (Figure 3), as in control cultures; basal [³⁵S]methionine incorporation into the cells during this period was similarly unaffected (results not shown). However, between 48 and 72 h, a lower rate of accumulation was exhibited by treated cells and at the end of this period their number was 47% of that observed in control cultures.

Morphological studies

The results shown in Figure 4 indicate that DNA in highly [³H]thymidine-labelled c-di-GMP-treated cells is maintained within the nuclei. [³H]Thymidine was added to 44 h c-di-GMP-preincubated cultures which were then fixed and autoradiographed. C-di-GMP-treated Molt 4 cells were heavily labelled (Figure 4b). Toluidine Blue staining of these cells clearly indicates that the positive heavy radioactive label is concentrated in the

Figure 4 [^3H]Thymidine incorporation into Molt 4 cells nuclei following incubation with c-di-GMP

Molt 4 cells grown for 48 h in the absence or presence of 50 μM c-di-GMP, 50 μM cyclic GMP or 50 μM GMP were pulsed with 1 μCi of [^3H]thymidine and then fixed with 4% glutaraldehyde and autoradiographed. The cells were then stained with Toluidine Blue. The dark nuclei identify those cells into which [^3H]thymidine was incorporated. Presented are: (a) cells grown in the absence of nucleotides (similar results were obtained in the presence of cyclic GMP or GMP); (b) cells grown in the presence of c-di-GMP.

nuclei of the cells. Between 70 and 90% of the cells were found to be involved in [^3H]thymidine incorporation. In contrast, untreated control cells (Figure 4a), or cells treated with cyclic GMP or GMP, showed a normal pattern of labelling. It is noteworthy that these results were obtained with cells grown on rich medium, supplemented by 10% (v/v) heat-inactivated foetal-calf serum, thus eliminating possible effects on cell growth rate and cellular metabolism, which may be caused by serum alterations.

Entrance of c-di-GMP to Molt cells

Since the above findings raise the possibility that c-di-GMP may have an activating effect on Molt 4 cells, we sought the possible mechanism by which c-di-GMP may exert its effect. Toward this goal, the entrance of c-di-GMP into the cells was first ascertained. Nucleotides extracted from Molt 4 cells, grown in the presence of [^{32}P]c-di-GMP and then extensively washed, were analysed by

Figure 5 Two-dimensional thin-layer chromatogram of extracts of [^{32}P]c-di-GMP-grown Molt 4 cells

Extracts of Molt 4 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 the Materials and methods section. 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.

two-dimensional TLC (using solvents A and B) and autoradiography for the presence of labelled c-di-GMP. Figure 5 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 analyses 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* [8,10,12,13]. These experiments strongly suggest that c-di-GMP enters Molt 4 cells. The concentration of free intracellular c-di-GMP was found to reach a value of 27 pmol per 10^6 cells, as determined by both TLC and enzymic analyses [8,10,13]. 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 $P < 0.001$.

The effect of c-di-GMP on [^3H]thymidine incorporation is irreversible and correlates with cellular c-di-GMP entrance

Cells pre-incubated for 4 h with c-di-GMP were washed three times, supplemented with fresh c-di-GMP-free medium and, after 40 h, further incubated for 4 h with [^3H]thymidine. The cells were then harvested. As seen in Figure 6(a), thymidine incorporation into these cells after 48 h was 12-fold higher than that of control cultures. Furthermore, when the intracellular nucleotides of such [^{32}P]c-di-GMP-treated cells, which were washed and harvested after 44 h, were extracted and analysed by two-dimensional TLC, [^{32}P]c-di-GMP could clearly be detected (Figure 6b).

C-di-GMP specifically binds to low-molecular-mass proteins in Molt 4 cells

Since c-di-GMP was found to enter Molt 4 cells, we sought to find out whether this novel nucleotide specifically binds to Molt

Figure 6 Thymidine incorporation and cellular c-di-GMP in c-di-GMP-pretreated Molt 4 cells

Molt 4 cells pre-incubated for 4 h with either 50 μM c-di-GMP (a) or $[^{32}\text{P}]\text{c-di-GMP}$ (b) were washed and supplemented with fresh c-di-GMP-free medium and then pulsed with $[^3\text{H}]$ thymidine and assayed, at the times indicated (a), or harvested and analysed by two-dimensional TLC (b) as described in the Materials and methods section. $[^3\text{H}]$ thymidine uptake in control cultures was 1300 c.p.m. at 24 h and 1700 c.p.m. at 48 h. Values represent means from at least three independent experiments, each performed in triplicate. S.E.M. was always less than 10% of the mean. $P < 0.001$ (a). (b) Presented is an autoradiogram of the exposed TLC plate. The large dark spot coincides with the position of a similarly chromatographed $[^{32}\text{P}]\text{c-di-GMP}$ marker.

Figure 7 C-di-GMP binding to Molt 4 cells proteins

Molt 4 cells were incubated for 48 h with 5 nM $[^{32}\text{P}]\text{c-di-GMP}$ in the absence or presence of various unlabelled nucleotides (lanes 1 to 8). Cells were then lysed, and proteins in the postnuclear supernatants were resolved by SDS/PAGE as described in the Materials and methods section. Presented is an autoradiogram of the exposed gels. Lane 1, cells incubated only with 5 nM $[^{32}\text{P}]\text{c-di-GMP}$; lane 2, 0.5 μM c-di-GMP added; lane 3, 1 μM c-di-GMP added; lane 4, 5 μM c-di-GMP added; lane 5, 5 μM GMP added; lane 6, 5 μM GDP added; lane 7, 5 μM GTP added [lower concentrations of GMP, GDP, GTP (10 nM–5 μM) had no effect on labelling]; lane 8, Molt 4 cells boiled prior to incubation with 5 nM $[^{32}\text{P}]\text{c-di-GMP}$ for 48 h; lane 9, Molt 4 cells incubated with 10 nM $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ for 48 h. Medium alone incubated with 5 nM $[^{32}\text{P}]\text{c-di-GMP}$ or 10 nM $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ for 48 h showed no labelling. Similarly analysed last cell wash prior to lysis showed no labelling.

4 cell proteins and may exert its effect via such binding. For this purpose, Molt 4 cells were incubated with $[^{32}\text{P}]\text{c-di-GMP}$ for 48 h, lysed and then subjected to SDS/PAGE and autoradiography. The data in Figure 7 show that at least two major and one minor different proteins, displaying apparent molecular masses of 18–27 kDa (lane 1), were indeed labelled from among the various proteins detected by Coomassie Blue staining. No labelling was detected employing heat-inactivated cells (lane 8), or when the medium and the last cell wash prior to lysis were similarly analysed (results not shown). Labelling of these proteins was gradually blocked by unlabelled c-di-GMP when present in 100-, 200- or 1000-fold molar excess (Figure 7, lanes 2–4). In contrast, unlabelled GMP, GDP, GTP (lanes 5–7) or cyclic GMP

(results not shown) at 1000-fold molar excess did not significantly interfere with the labelling by $[^{32}\text{P}]\text{c-di-GMP}$ (less than 5% as quantified by scanning densitometry of the labelled proteins, results not shown). Although c-di-GMP may enzymically be hydrolysed to GMP (via the linear dinucleotide pG3'p5'G) [8], the inability of GMP, GDP and GTP to effectively compete with the labelling by c-di-GMP of the 18–27 kDa proteins strongly supports our conclusion that the intensive labelling observed is indeed due to c-di-GMP binding to these proteins and not to guanosine phosphates 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.

GTP binds at a lower intensity to similar low-molecular-mass proteins in Molt 4 cells

With c-di-GMP being a guanine nucleotide, and since proteins in the range of 18–27 kDa of various eukaryotic systems [14,15] are known to bind GTP, we repeated the above-noted experiment by incubating cells with $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ at twice the molar concentration and total radiolabelling of the c-di-GMP employed. As seen in Figure 7 (lane 9) a similar pattern of labelled proteins was observed. However, in spite of the lower molar concentration of the c-di-GMP relative to the GTP employed in these experiments, the labelling intensity of the 21 kDa protein observed with $[^{32}\text{P}]\text{c-di-GMP}$ is considerably higher than that obtained with $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ (Figure 7, lane 1 versus lane 9). Indeed, when the 21 kDa bands of the two experiments were cut out and their radioactivity counted, the labelling of the protein in the c-di-GMP experiment was 20-fold greater than that obtained with GTP. Similar results were obtained by scanning densitometry of the autoradiograms.

C-di-GMP binds to the p21^{ras} growth-promoting protein

Given the central role of the 21 kDa G-protein from the Ras superfamily in transducing signals downstream, resulting in transformation and proliferation in various types of cell lines [16], we deduced that p21^{ras} might be the 21 kDa protein to which c-di-GMP binds. To demonstrate this, Molt 4 cells previously incubated for 48 h with either $[^{32}\text{P}]\text{c-di-GMP}$ or $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ were lysed and then immunoprecipitated with the anti-Ras monoclonal antibody Y13-259. The precipitates were subjected to SDS/PAGE analysis and autoradiography. Figure 8 shows that in both immunoprecipitates (lanes 1 and 7), a labelled 21 kDa band was observed. Such a labelled band was not seen when Y13-259 was substituted by rat anti-(mouse IgG) monoclonal antibody (lanes 6 and 8). Labelling of the p21^{ras}-Y13-259 complex by $[^{32}\text{P}]\text{c-di-GMP}$ was not significantly interfered with (less than 7% as quantified by scanning densitometry, results not shown) in the presence of a 1000-fold molar excess of unlabelled GTP, GDP and GMP (Figure 8, lanes 3–5) or cyclic GMP (results not shown). In contrast, a 1000-fold molar excess of unlabelled c-di-GMP completely blocked the labelling of the immunoprecipitated protein (Figure 8, lane 2). The results of these competition experiments further suggest that the intensive labelling obtained with $[^{32}\text{P}]\text{c-di-GMP}$ is due to the binding of the latter to p21^{ras} rather than to the binding of any guanosine phosphates which may be generated from the cyclic dinucleotide. It can clearly be seen that, in this case too, the label intensity of the 21 kDa protein immunoprecipitated from $[^{32}\text{P}]\text{c-di-GMP}$ -treated cells is considerably higher (at least 20-fold by counting) than that precipitated from $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ -treated cells.

Figure 8 SDS/PAGE analysis of immunoprecipitates of p21^{ras} from lysates of Molt 4 cells labelled with [³²P]c-di-GMP or [α -³²P]GTP

Molt 4 cells incubated with either 5 nM [³²P]c-di-GMP (0.8 Ci/ μ mol; lanes 1–6) or 10 nM [α -³²P]GTP (0.8 Ci/ μ mol; lanes 7 and 8) in the absence or presence of various unlabelled nucleotides (lanes 1, 6–8, no nucleotide added; lane 2, 5 μ M c-di-GMP added; lane 3, 5 μ M GTP added; lane 4, 5 μ M GDP added; lane 5, 5 μ M GMP added; lower concentrations of GTP, GTP and GMP had no effect on labelling) were lysed and immunoprecipitated as described in the Materials and methods section with the following antibodies: lanes 1–5 and 7, Ras-specific monoclonal antibody Y13-259; lanes 6 and 8, rat anti-(mouse IgG) monoclonal antibody.

DISCUSSION

This study reports on the specific binding of the novel, naturally occurring regulatory nucleotide cyclic di-GMP to the p21^{ras} protein and its ability to markedly stimulate DNA synthesis in the Molt 4 cell line. The dramatic effect on thymidine uptake by these cells (Figures 2a and 2b) appears to be unique to c-di-GMP and did not occur when other guanine nucleotides, including stable GTP analogues such as GTP[S] and p[NH]ppG, were used. The differences observed between the extent of increase in [³H]thymidine incorporation (200-fold over control), the increase in DNA content (9-fold) and the increase in the specific radioactivity of acid-precipitable [³H]thymidine-labelled DNA suggest that c-di-GMP affects thymidine uptake into the cells, and possibly also the relative usage of *de novo* synthetic and salvage pathways. Another possibility is that the DNA is damaged in the treated cells, with the result that repair synthesis is occurring.

Guanine nucleotides have long been reported to interact with the GTP-binding p21^{ras} protein. Injection of p21^{ras} to NIH3T3 cells has been reported to result in increased thymidine uptake, accompanied by morphological changes and increased cell division [17]. At variance with the latter report, treatment of Molt 4 cells with c-di-GMP under the conditions described here, although strikingly elevating DNA synthesis, had no demonstrable effect on either cell replication (up to 48 h) or cellular [³⁵S]methionine uptake, nor on morphological transformations as checked by electron-microscopy (results not shown). These findings raise the possibility 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. This phenomenon may be a result of the nature of the c-di-GMP–p21^{ras} binding complex. Since the binding of c-di-GMP to p21^{ras} is apparently specific and cannot be blocked by other guanine nucleotides (Figure 8, lanes 3–5); also since it remains attached to p21^{ras} after boiling (Figures 7 and 8), suggesting that they are covalently linked, it appears that the cyclic nucleotide binds irreversibly to p21^{ras}, thereby maintaining it in a fixed active conformation. This possibility is compatible with the irreversible nature of the c-di-GMP effect on DNA synthesis observed even after removal of external c-di-GMP from c-di-GMP-treated cells. The overall c-di-GMP effects become toxic after 48 h of culture, as is evident by the decline after 72 h in the number of treated cells relative to control cultures (Figure 3).

Accumulation of the Ras–GTP conformation has been observed in activated T-lymphocytes [1,18]. Our experiments (Figure 7) indicate that labelling of the 21 kDa protein by [³²P]c-di-GMP is 20-fold higher than the labelling of the protein by [α -³²P]GTP. Similar results were obtained in immunoprecipitation studies employing monoclonal antibodies against the Ras protein (Figure 8). The difference in signals observed with the labelled dinucleotide and GTP may be due to the possibility that GTP is metabolized more quickly and to a greater extent than c-di-GMP after entering the cells.

Independent of the arguments mentioned above, since c-di-GMP can be hydrolysed only by a specific phosphodiesterase and not by GTPase, the GTPase activating protein mechanism might be inhibited, thereby allowing c-di-GMP to be continuously bound to p21^{ras}. On the other hand, the nucleotide exchange reaction [4–6,19–21] might not occur, due to the inability of the system to recognize the c-di-GMP–Ras complex and, therefore, p21^{ras} would not be free to bind GTP. Such disruption in the delicate dynamic balance between the two mechanisms could result in a constitutive stable c-di-GMP–p21^{ras} complex that would remain in the ‘active’ conformation.

The effects of c-di-GMP described in this report were observed under conditions which we feel avoid any cell wall alterations that can be caused following injection or permeabilization techniques [1–3,17,22]. Furthermore, the effects were obtained with cells grown for 48 h in 10% foetal-calf serum-containing medium, providing optimal conditions for cell growth and proliferation, thereby avoiding the deleterious effects on cell fragility, viability, growth rate and metabolism reportedly associated with changes in medium serum levels [23–25]. It appears to be of special significance that, under our experimental conditions, c-di-GMP and GTP freely enter the Molt 4 cells and, as seen in Figure 5, free c-di-GMP can indeed be found within the cells.

Although remote, the possibility that the labelling of p21^{ras} and other small proteins is due to their phosphorylation by c-di-GMP via an as yet unknown mechanism cannot be ruled out. Of the three major different labelled proteins observed in [³²P]c-di-GMP-treated cells, the p21^{ras} was identified by the anti-Ras monoclonal antibody, Y13-259. However, the possibility that the 21 kDa band contains additional proteins cannot be excluded [26,27]. The identity of the other proteins displaying apparent molecular masses of about 18 and 27 kDa remains to be established.

In conclusion, the naturally occurring guanine nucleotide, c-di-GMP, enters Molt 4 cells and, subsequently, can be found intracellularly free and intact. Notably, its entrance does not require permeabilization of the cells. Once inside, the guanine nucleotide binds to p21^{ras} and possibly maintains the proto-oncogenic protein in a fixed active state, which may cause the dramatic stimulation in DNA synthesis observed. Of special significance is the fact that the latter effect is not accompanied by increased cell proliferation or morphological changes in the cell and is not due to serum-level alterations. In addition, c-di-GMP specifically binds to other low-molecular-mass proteins.

Taking all the data presented here into consideration, we feel that our system can offer an attractive model for the study of cell cycle-involved proteins and Ras trans-signalled downstream proteins. Although Ras-dependent function is now outlined in part, elucidation of the finer details of the signalling network might be facilitated by our experimental system.

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