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In vitro evidence for the recognition of 8-oxoGTP by Ras, a small GTP-binding protein

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Abstract

Oxygen radicals attack guanine bases in DNA but they also attack cytoplasmic GTP forming 8-oxoGTP. The presence of 8-oxoGTP in cytoplasm is evidenced by the fact that cells contain MutT/MTH1 which hydrolyze 8-oxoGTP into 8-oxoGMP. In this study, the interaction between 8-oxoGTP and Ras, a small GTP-binding protein, was tested in vitro, and the action of 8-oxoGTP was compared to that of GTP. When purified Ras was treated with 8-oxoGTPγS, Ras was activated, as indicated by the enhanced binding of Ras with Raf-1. GTPγS also activated Ras but 8-oxoGTPγS had a much more potent effect. In lysates of human embryo kidney 293 cells, 8-oxoGTPγS activated not only Ras but also the downstream effectors of the Ras-ERK pathway, i.e., Raf-1 and ERK1/2. In contrast to Ras, other small GTP-binding proteins, Rac1 and Cdc42, were inactivated by 8-oxoGTPγS, whereas both of these proteins were activated by GTPγS, indicating that the biological natures of 8-oxoGTP and GTP differ. These results suggest the possibility that 8-oxoGTP is not a simple by-product but a functional molecule transmitting an oxidative signal to small GTP-binding proteins like Ras.

Keywords: Oxidative stress; 8-oxoGTP; Ras; Small GTP-binding protein

Aerobic organisms cannot avoid oxidative stress because reactive oxidative species (ROS) are generated by the normal cellular metabolism. ROS have been implicated in carcinogenesis by directly damaging DNA and by acting as tumor promoters [1,2]. In addition, ROS have been identified as central mediators in certain signaling events. Extracellular signal-regulated kinase (ERK) is one of the ROS-response mitogen-activated protein kinases (MAPKs) [3,4]. Moreover, a growing body of evidence suggests that the small GTP-binding protein, Ras, has an essential role in the oxidative signal transduction pathway; more specifically, Ras seems to be involved in the activation of ERK by ROS [5,6]. However, the precise mechanisms of Ras activation by ROS, through which Ras affects its downstream targets, are not known.

One of the most common DNA lesions generated by ROS is an oxidized guanine (8-oxoguanine; 8-oxoG), which can pair with adenine in DNA and cause transversion mutation [7–9]. However, a considerable amount of 8-oxoG is generated in the cellular nucleotide pool as well as in DNA by ROS because the nucleotide pool is mainly located in the cytoplasm, and the cytoplasmic nucleotides can be more easily attacked by ROS than DNA in the nucleus. Furthermore, the pool size of

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cellular ribonucleotides is hundreds of times larger than that of deoxyribonucleotides [10], and therefore, significant amounts of 8-oxoG-containing ribonucleotides like 8-oxoGTP are probably produced in the cytoplasm.

Actually, 8-oxoGTP was found to be incorporated into RNA and to cause translational errors in *Escherichia coli* [11]. Accordingly, organisms are equipped with elaborate mechanisms to eliminate such oxidized nucleotides from the nucleotide pool; *E. coli* and humans possess MutT and MTH1, respectively, which hydrolyze and remove oxidized nucleotides [12–14]. In addition, MTH1 activity was found to be located primarily in the cytoplasmic fraction of human Jurkat cells [15]. More recently, mammalian YB-1 protein was reported to bind specifically to RNA containing 8-oxoG and to promote the fidelity of translation [16]. Although cellular defense mechanisms have been evolved to manage the deleterious effects of cytoplasmic 8-oxoGTP like this, studies on 8-oxoG have largely focused upon its mutagenic effects in DNA.

In fact, GTP is involved in many intracellular processes as well as in RNA synthesis; these include energy metabolism, microtubule assembly, protein synthesis, and cell signaling. Accordingly, the conversion of GTP into 8-oxoGTP by oxidative stress may affect the above cellular process. Among these various possibilities, we hypothesized that 8-oxoGTP may directly act on an oxidative sensor, possibly Ras, to influence Ras-ERK activity. Because Ras, a master controller of the central cellular signaling pathways, is a small GTP-binding protein, 8-oxoGTP, the oxidized form of GTP, may function differently from normal GTP.

Therefore, the present study was undertaken to examine the interaction between Ras and 8-oxoGTP in vitro, by observing whether 8-oxoGTP affects the activities of Ras and ensuing downstream kinases. The results obtained demonstrate that Ras is activated by 8-oxoGTP and that this leads to the activation of downstream kinases. It is hoped that the in vitro data provide an insight to how ROS modulate cellular functions.

Materials and methods

Cells and reagents. Human embryo kidney 293 (HEK293) cells were cultured in a humidified 5% CO_2 atmosphere at 37 °C in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. When cells were at an exponential phase, they were starved in serum-free DMEM overnight, harvested, and used in the experiments. 8-oxoGTPγS (8-oxoguanosine 5′-3-*O*-[thio]triphosphate) and GTPγS (guanosine 5′-3-*O*-[thio]triphosphate), non-hydrolyzable analogs of GTP, were custom-made by Trilink Biotechnologies (San Diego, USA). GDP was obtained from Sigma (St. Louis, USA).

Ras activation (Ras-Raf-1 binding) assay. This assay was performed as described previously [17,18] using a Ras activation kit (Upstate Biotechnology, Lake Placid, USA). Purified recombinant Ras was obtained from Calbiochem (San Diego, USA) and resuspended in a magnesium-containing lysis buffer (MLB; 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1% Igepal CA-630, 2% glycerol, 10 μg/μl aprotinin, 10 μg/μl leupeptin, 25 mM NaF, 1 mM Na_3VO_4, and 25 mM Hepes, pH 7.5). To prepare cell lysates, the harvested HEK293 cells were washed twice with PBS, treated with MLB on ice for 30 min, and centrifuged at 13,000*g* for 15 min. Supernatants were collected as lysates and protein concentrations were determined. The purified Ras (0.1 μg) or cell lysate (500 μg protein) was treated with GDP, GTPγS or 8-oxoGTPγS of concentrations indicated in 1 ml MLB containing 10 mM EDTA for 15 min at 30 °C. To stabilize the binding between Ras and guanine nucleotides, $MgCl_2$ was added to a final concentration of 60 mM and cooled on ice. To collect the activated Ras, the reactions were mixed with 5 μg GST-Raf-1-RBD (a fusion protein of glutathione-*S*-transferase and Raf-1 Ras-binding domain) conjugated to glutathione–agarose beads at 4 °C for 1 h and the beads were washed with MLB three times. The beads were resuspended in 20 μl of 2× SDS sample buffer, boiled for 5 min, and electrophoresed in 10% SDS–PAGE. The gels were transferred onto nitrocellulose membranes and Ras in the membranes was detected by Western blotting with a mouse monoclonal anti-Ras antibody in the kit. The band densities were quantified using a BAS 2500 imaging analyzer (Fuji Photo Film, Japan).

Raf-1 kinase assay. Raf-1 kinase assay was performed as described previously [19]. Briefly, aliquots (400 μg protein) of HEK293 cell lysate prepared in the previous section were reacted with GDP (1 mM), GTPγS (100 μM) or 8-oxoGTPγS (100 μM) in 1 ml MLB containing 10 mM EDTA for 15 min at 30 °C and then mixed with 2 μg of a rabbit polyclonal anti-Raf-1 antibody (Upstate Biotechnology, Lake Placid, USA) for 90 min at 4 °C. After adding 30 μl protein A–agarose (Oncogene, Boston, USA), the reactions were allowed for 90 min at 4 °C. The immune complex bound to the agarose was washed with MLB three times and with kinase buffer (10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, 5 μM ATP, and 25 mM Hepes, pH 7.4) twice, and then incubated in 40 μl of the kinase buffer containing 20 μCi [γ-^{32}P]ATP (Amersham Biosciences, Uppsala, Sweden) and 1 μg MEK-1 (Santa Cruz Biotechnology, Santa Cruz, USA) as a substrate for 30 min at 30 °C. The reactions were terminated by adding 4× SDS sample buffer (15 μl) and subjected to 8% SDS–PAGE and autoradiography. In order to measure the amounts of Raf-1 and MEK-1 from each reaction, the electrophoretic gels were also transferred onto nitrocellulose membranes and Western blottings were performed with a rabbit polyclonal anti-Raf-1 antibody (Upstate Biotechnology, Lake Placid, USA) and a rabbit polyclonal anti-MEK1/2 antibody (Cell Signaling, Beverly, USA), respectively.

ERK1/2 activation assay. The terminal effectors of Ras-ERK pathway are ERK1/2 (ERK1 and ERK2 are 44- and 42-kDa proteins, respectively). When phosphorylated on their tyrosine and threonine residues, these proteins are activated. When the lysate of HEK293 was treated with GTPγS or 8-oxoGTPγS, the phosphorylated ERK1/2 (pERK1/2) were barely detected probably due to the disruption of natural arrangement of effector proteins in the Ras-ERK pathway. Thus, we decided to inject the guanine nucleotides into HEK293 cells and to observe pERK1/2 from the lysates of the nucleotide-injected cells. For this purpose, 2×10^5 HEK293 cells were electroporated as previously described [20] in DMEM containing GDP (1 mM), GTPγS (100 μM) or 8-oxoGTPγS (100 μM). After 20 min incubation at 37 °C, cell lysates were prepared as described in the Ras activation assay section. The cell lysates were electrophoresed in a 10% SDS–PAGE and transferred onto a nitrocellulose membrane. Blots were probed with a rabbit polyclonal anti-ERK1/2 or anti-pERK1/2 (Cell Signaling Technology, Beverly, USA).

Rac1 and Cdc42 (Rac1/Cdc42-PAK-1-PBD binding) activation assay. The activation assays of the two small GTP-binding proteins, Rac1 and Cdc42, were performed according to the method described previously [17,21], using Rac1 and Cdc42 activation kits (Upstate Biotechnology, Lake Placid, USA). But the experimental procedures were basically the same with the Ras activation assay procedures. Purified recombinant Rac1 and Cdc42 were obtained from Calbiochem (San Diego, USA) and resuspended in MLB and lysates of HEK293

cells were prepared using MLB as described in Ras activation assay section. The purified Rac1, Cdc42 (0.1 μg, each) or cell lysate (500 μg protein) was treated with GDP, GTPγS or 8-oxoGTPγS of concentrations indicated in 1 ml MLB containing 10 mM EDTA for 15 min at 30 °C and then $MgCl_2$ was also added to a concentration of 60 mM and cooled on ice. The activated Rac1 or Cdc42 was affinity-precipitated by adding 5 μg GST-PAK-1-PBD (a fusion protein of glutathione-*S*-transferase and p21-activated protein kinase 1 p21-binding domain) conjugated to glutathione–agarose beads at 4 °C for 1 h. The following procedures were exactly the same as in the Ras activation assay but Rac1 and Cdc42 were probed with a mouse monoclonal anti-Rac1 or anti-Cdc42 antibody.

Results

Effect of 8-oxoGTP on the Ras activity

Fig. 1A shows that 8-oxoGTPγS activates Ras and that its ability in this respect is even greater than that of GTPγS in both purified recombinant Ras and cell lysates. These in vitro results indicate that 8-oxoGTP is a more potent activator of Ras than GTP. Using purified recombinant Ras, we compared the potencies of 8-oxoGTPγS and GTPγS at various concentrations (Fig. 1B). As shown in Fig. 1B, Ras activity was enhanced by increasing 8-oxoGTPγS or GTPγS, and the enhancement of Ras activity by 8-oxoGTPγS was greater than that by GTPγS at all concentrations used. The difference between enhancement by 8-oxoGTPγS or GTPγS increased with increasing their concentration, and at concentrations higher than 80 μM, enhancement by 8-oxoGTPγS was 1.5 to twice that of GTPγS. 8-oxoGTP formed from GTP might exist with GTP in cytoplasm. Therefore, under the physiological conditions, Ras is expected to be exposed to both 8-oxoGTP and GTP. With this in mind we examined the effects of the mixtures of 8-oxoGTPγS and GTPγS on Ras activity. Mixtures were prepared containing various concentration combinations of the two, though their total concentration was held at 100 μM. As shown in Fig. 1C, the activation of Ras appears to depend on the proportion of 8-oxoGTPγS in both purified Ras and cell lysates, and maximum activation was observed for 75 μM of 8-oxoGTPγS and 25 μM GTPγS. Moreover, it should be noted that the effect of the 75:25 mixture was even greater than that of 100 μM 8-oxoGTPγS alone, which suggests that 8-oxoGTP and GTP may act synergistically in cytoplasm.

Effects of 8-oxoGTP on the activities of the downstream effectors of ras-ERK pathway

After confirming the activation of Ras by 8-oxoGTPγS, we also observed the effects of this binding on the downstream effectors of the Ras-ERK pathway. First, Raf-1 kinase, which is activated by binding with Ras, was assayed using MEK-1 as a substrate.

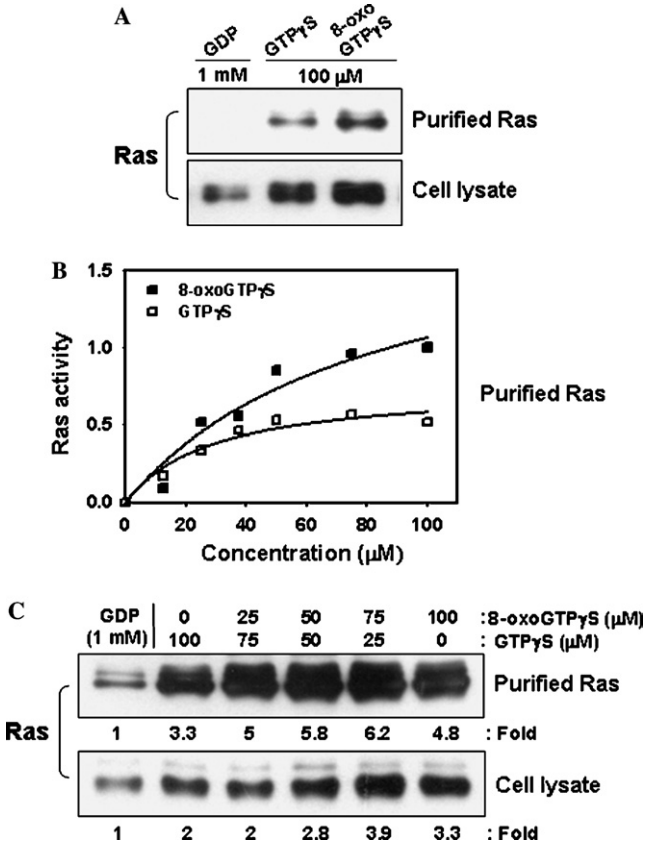

Fig. 1. Activation of Ras by 8-oxoGTP. Purified recombinant Ras was resuspended in a magnesium-containing lysis buffer (MLB; see Materials and methods) and lysate of HEK293 cells was prepared and resuspended in MLB as described in Materials and methods. Purified Ras (0.1 μg) or cell lysate (500 μg protein) was incubated with GDP, GTPγS or 8-oxoGTPγS of various concentrations indicated in 1 ml MLB containing 10 mM EDTA for 15 min at 30 °C and then $MgCl_2$ was added to a concentration of 60 mM to stabilize the binding between Ras and guanosine nucleotides. The reactions were mixed with 5 μg GST-Raf-1-RBD conjugated to glutathione–agarose beads for 4 °C for 1 h. The beads which have precipitates of Ras/Raf-1-RBD were collected, boiled in 2× SDS sample buffer, and electrophoresed in 10% SDS–PAGE. The gels were transferred onto nitrocellulose membranes, which were probed with a mouse monoclonal anti-Ras antibody. Details are described in Materials and methods. (A) Activation of Ras by 8-oxoGTPγS and comparison of its effect to that of GTPγS, (B) Ras activation at various concentrations of GTPγS and 8-oxoGTPγS, and (C) Ras activation by various concentration combinations of GTPγS and 8-oxoGTPγS.

As shown in Fig. 2, Raf-1 kinase was activated by 8-oxoGTPγS and GTPγS, and this activation by 8-oxoGTPγS was about 2.5-fold of that by GTPγS. The densities of GTPγS- and 8-oxoGTPγS-treated bands were compared after subtracting the background density of each lane from the respective band densities since the phosphorylated band of MEK-1 was more intense in the lane treated with 8-oxoGTPγS, but so was the background.

We found that 8-oxoGTPγS is a more potent stimulator than GTPγS on the first two effectors, Ras and Raf-1. Thus, we examined whether this difference was observa-

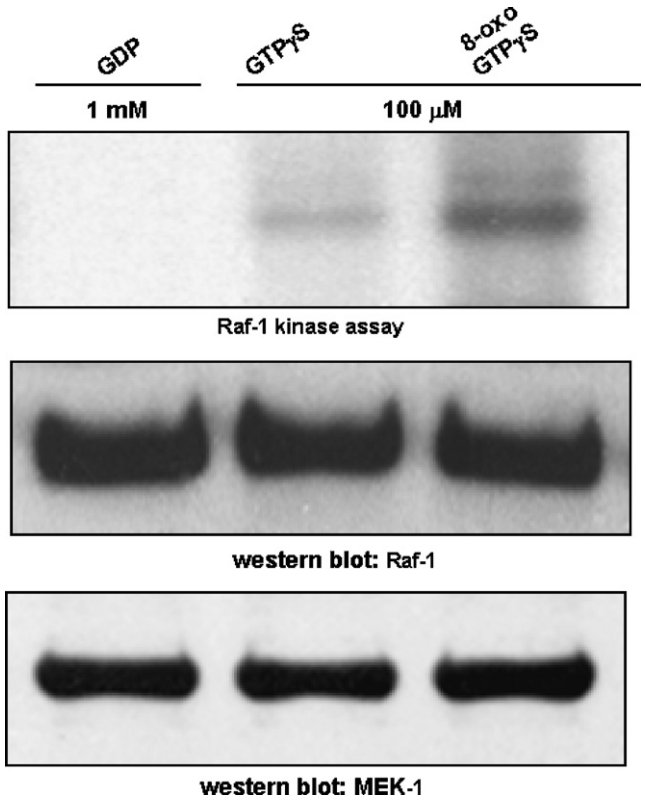

Fig. 2. Activation of Raf-1 by 8-oxoGTP. The preparation of HEK293 lysate and treatment of the lysate (400 μg) with GDP, GTPγS or 8-oxoGTPγS were carried out as in Fig. 1. The reactions were mixed with 2 μg of a mouse monoclonal anti-Raf-1 antibody for 90 min at 4 °C and then mixed with 30 μl protein A–agarose for 90 min at 4 °C. The immune complex bound to the agarose was washed with MLB three times and with kinase buffer (see Materials and methods) twice and then incubated for 30 min at 30 °C in 40 μl of the kinase buffer containing 20 μCi [γ-^{32}P]ATP and 1 μg MEK-1 as a substrate. The reactions were terminated by adding 4 SDS sample buffer (15 μl) and subjected to 8% SDS–PAGE and autoradiography (top panel). In order to measure the amounts of Raf-1 and MEK-1 from each reaction, the electrophoretic gel was also transferred onto a nitrocellulose membrane, which was probed with a rabbit polyclonal anti-Raf-1 antibody (middle panel) and a rabbit polyclonal anti-MEK1/2 antibody (bottom panel).

ble at the level of ERK, the most downstream effector of the Ras-ERK pathway. Accordingly, we incubated HEK293 cell lysates with GDP, GTPγS, or 8-oxoGTPγS, and then measured the amounts of ERK1/2 and phosphorylated ERK1/2 (pERK1/2) by Western blotting using anti-ERK1/2 and anti-pERK1/2 antibodies. However, the effect of either 8-oxoGTPγS or GTPγS was not observed clearly at this level. We believe that this was due to the disturbance of the optimal assembly of the effector proteins of Ras-ERK pathway in the lysate. As ERK is a terminal protein in this signaling pathway, it may thus be most susceptible to the assembly disturbance. To overcome this problem derived from the use of cell lysate for ERK activity assay, we decided to inject each of the GDP, GTPγS, or 8-oxoGTPγS into cells. For this purpose, HEK293 cells were electroporated with

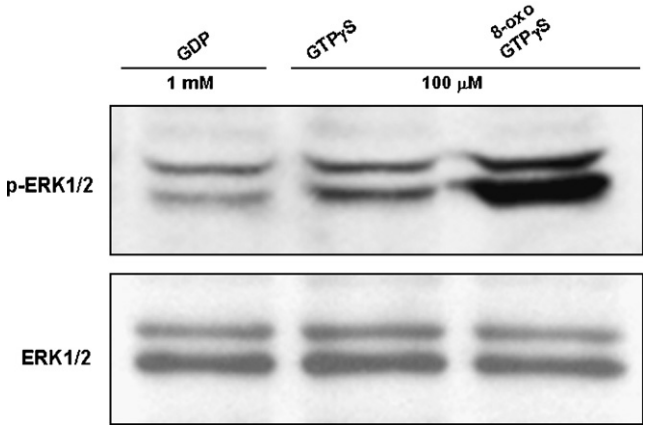

Fig. 3. ERK1/2 activation by 8-oxoGTP. The effects of guanosine nucleotides were observed after they were injected into HEK293 cells because their effects were hardly observed when lysates of HEK293 were treated with these compounds. Thus, HEK293 cells were electroporated in DMEM with guanosine nucleotides of concentrations indicated and incubated for 20 min at 37 °C. Cell lysates were prepared as in Fig. 1 and subjected to 10% SDS–PAGE. The gels were transferred onto nitrocellulose membranes. Blots were probed with rabbit polyclonal anti-phospho ERK1/2 (p-ERK1/2) and anti-ERK1/2 antibodies. Details are described in Materials and methods.

each of the guanine nucleotides and then lysed after being incubated for 20 min. The amounts of ERK1/2 and pERK1/2 were measured by Western blotting the lysates, as described above. Under this in vivo condition used, we observed the effects of GTPγS and 8-oxoGTPγS, and also found that 8-oxoGTPγS is more potent than GTPγS as indicated by the band densities (Fig. 3).

Effects of 8-oxoGTP on other small GTP-binding proteins, Rac1 and Cdc42

In addition to Ras, there are many other kinds of small GTP-binding proteins in the Ras superfamily. These small proteins have enormous functional diversity, about which much is unknown, but like Ras they too have a GTP-binding site. Thus, we undertook to determine how the oxidation of the 8 position of guanine affects its binding with other members in the Ras superfamily. For this purpose, we examined the effect of 8-oxoGTPγS on Rac1 (Fig. 4A and B) and Cdc42 (Fig. 4C) using purified recombinants or cell lysate (Fig. 4). These two proteins are members of the Rho family of small GTP-binding proteins [22]. These two proteins link to and activate PAK-1 (p21-activated kinase 1) in the same manner as the Ras-Raf-1 interaction. Using GST-PAK-1-PBD conjugated to glutathione–agarose beads, which contains Cdc42- and Rac1-binding site [21], the effect of 8-oxoGTP was tested with respect to the activation of these two proteins. In contrast to Ras, Rac1, and Cdc42 were not activated by 8-oxoGTPγS though they were strongly activated by

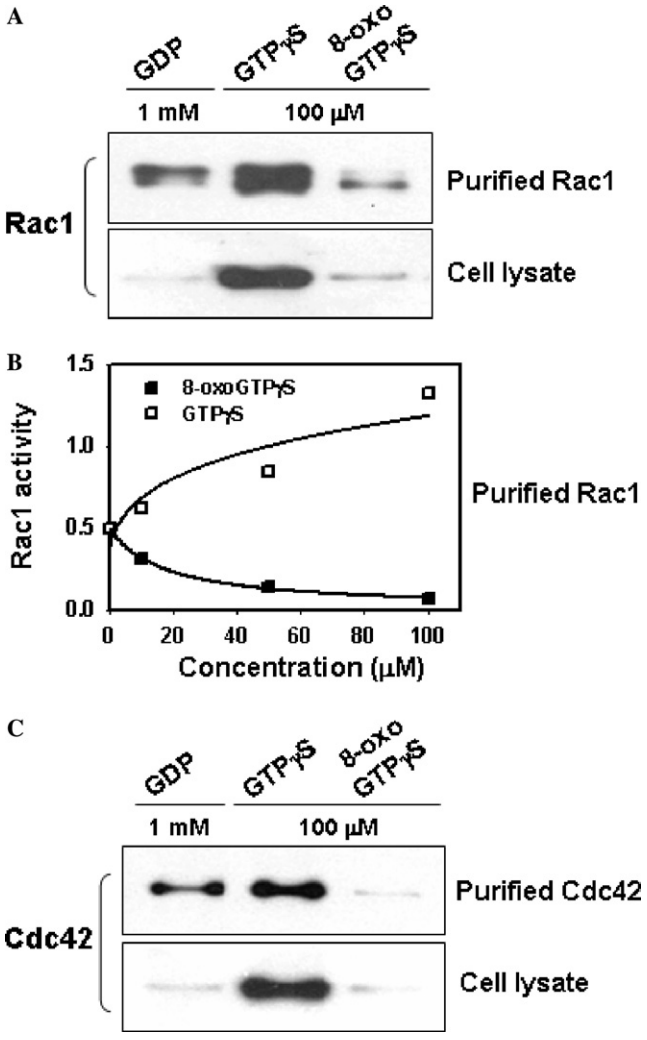

Fig. 4. Inactivation of Rac1 and Cdc42 by 8-oxoGTP. The experimental procedures were exactly the same as in Fig. 1, except the replacement of purified recombinant Ras, GST-Raf-1-RBD conjugated to glutathione–agarose and mouse monoclonal anti-Ras antibody by purified recombinant Rac1 and Cdc42, GST-PAK-1-PBD conjugated to glutathione–agarose and mouse monoclonal anti-Rac1 and anti-Cdc42 antibodies, respectively. The same experimental procedures were applied to the lysates of HEK293 which were also prepared as in Fig. 1. The concentrations of guanine nucleotides used are shown in the figure. (A) Response of Rac1 to guanine nucleotides, (B) effects of various concentrations of GTPγS and 8-oxoGTPγS on Rac1 activity, and (C) response of Cdc42 to guanine nucleotides.

GTPγS. 8-oxoGTPγS behaved like GDP in terms of its interaction with these two proteins (Fig. 4). These results indicate that 8-oxoGTP acts differentially depending on the nature of the small GTP-binding protein involved.

Discussion

This study was performed to obtain in vitro evidence for the interaction between Ras and 8-oxoGTP as opposed to GTP. For this purpose, the non-hydrolyzable GTP analogs, GTPγS or 8-oxoGTPγS, were incubated with purified recombinant Ras or cell lysates prepared from the cell line, HEK293. Ras activity was then assayed by measuring its binding to Raf-1 using GST-Raf-1-RBD conjugated to glutathione–agarose beads. When Ras is activated by various stimuli, it can bind Raf-1, which is then activated. Therefore, the Ras activation by binding between Ras and Raf-1 is an initiating step that leads to Ras-ERK pathway activation. This in vitro study clearly shows that 8-oxoGTP activates Ras (Fig. 1) and that the Ras activation leads to the activation of this signaling pathway as indicated by the enhancement of Raf-1 activity (Fig. 2) and the increase in the amount of phosphorylated ERK (Fig. 3). In these actions, 8-oxoGTP was potent than GTP. However, Rac1 and Cdc42 were strongly inactivated by 8-oxoGTPγS while these proteins were activated by GTP (Fig. 4) indicating that 8-oxoGTP and GTP behave differently with respect to small GTP-binding protein.

It should be mentioned here that in the sample of Fig. 3 treated with GDP, there was a strong signal of pERK in quite a contrast to the results shown in Figs. 1 and 2, where there was no Ras binding or no Raf-1 activity in the presence of GDP. This result is opposed to our claim that there is some correlation or dependence of each downstream activity on 8-oxoGTP binding to Ras. The reason for these irregular responses to GDP is not known despite efforts to resolve this problem. However, we think that this is at least not due to the experimental conditions where the experiments in Figs. 1 and 2 were performed in vitro and those in Fig. 3 were performed in vivo since in Fig. 1, purified Ras showed no response to GDP but cell lysate showed a strong response to it and in Fig. 4, purified Rac1 and purified Cdc42 showed a clear response to GDP but in lysates, these two proteins showed almost no response to this chemical. The purpose of the present study was to determine the response of Ras to 8-oxoGTP, and the activity difference between 8-oxoGTP and GTP. Therefore, the data were presented as obtained but effects of GTPγS and 8-oxoGTPγS were compared with the band densities in the 8-oxoGTPγS and GTPγS lanes after subtracting the band densities in the GDP-treated lane.

According to a study on the binding affinities of nucleotides to Ras [23], substantial modifications of the sugar or phosphate structure of GTP are tolerated with little or only moderate affinity loss between these modified guanine nucleotides and Ras. However, the modifications of its base structure severely affect its affinity for Ras. This suggests that base structure is important for the binding of nucleotides to Ras. 8-Oxoguanine exists predominantly as a 6,8-di-keto tautomer, which has a *syn*-conformation rather than the more common *anti*-conformation [24]. Therefore, oxidation at the 8 position of guanine may not be a simple modification, and may be sufficient to induce an overall

conformational change sufficient to affect its binding to Ras, probably in the direction of increasing Ras affinity.

In this study, we obtained in vitro evidence that Ras is activated by 8-oxoGTP and that, in this respect, 8-oxoGTP is more potent than GTP. The meaning of this in vitro finding is not known. Many studies have shown that ROS induce various cellular responses; however, the detailed modes of ROS action with respect to these responses are also unknown. Nevertheless, our finding cautiously suggests the possibility that 8-oxoGTP mediates these oxidative responses. 8-oxoGTP, a small and rapidly diffusible molecule, may be easily generated by ROS and rapidly destroyed by MutT/MTH1. In other words, 8-oxoGTP may be a cellular molecule whose cytoplasmic level is rapidly changed by oxidative stress. Such reasoning strongly suggests that this molecule is not a simple by-product formed by oxidative stress but a functional molecule that acts on Ras or other small GTP-binding proteins.

To prove this hypothesis, however, many efforts remain to be done. We should first monitor the intracellular level of 8-oxoGTP, and then investigate whether 8-oxoGTP is formed at a sufficient level to influence the Ras activity. But the method of 8-oxoGTP assay has not been established yet. In addition, we should study how its action is terminated and finally identify the cellular responses induced by 8-oxoGTP in vivo. Regarding the termination of 8-oxoGTP action, one mechanism would be hydrolysis by MutT/MTH1, but whether it is hydrolyzed by GTPase of Ras protein remains to be determined. Now the X-ray crystallographic study is undertaken using a purified Ras to understand the direct binding nature of 8-oxoGTP to Ras. Although, the above appears preemptive, the results of the present study may provide a clue as to how oxidative signals trigger cellular responses.

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