The Ras GDP/GTP cycle is regulated by oxidizing agents at the level of Ras regulators and effectors

Katia Accorsi[a,b,1], Carmela Giglione[a,1,2], Marco Vanoni[b,*], Andrea Parmeggiani[a,3]

[a] *Groupe de Biophysique-Equipe 2, Ecole Polytechnique, F-91128 Palaiseau Cedex, France*
[b] *Dipartimento di Biotecnologie e Bioscienze, Universitá degli Studi di Milano-Bicocca, Piazza della Scienza no. 2, I-20126 Milan, Italy*

Received 12 January 2001; revised 12 February 2001; accepted 14 February 2001

First published online 23 February 2001

Edited by Matti Saraste

Abstract Reactive oxygen species (ROS) have been found to play important roles in regulating cellular functions. Their action in vivo has been related to specific effects on signal transduction pathways, such as Ras pathway. In order to characterize which elements of Ras pathway are affected by ROS, we have analyzed the action of different oxidizing agents on the ability of GTPase activating protein GAP and nucleotide exchange factor GEF to enhance the intrinsic activities of Ras. The action of these agents on the binding between H-Ras and its effector c-Raf-1 was also investigated. No effects were observed on the intrinsic activities of H-Ras or Ras2p. On the other hand, reversible inhibitions of GEF and GAP actions on Ras were found, whose extent was dependent on the agent used. As tested with the scintillation proximity assay, these agents also inhibited the binding of c-Raf-1 to H-Ras. Our data reveal new potential targets for the action of ROS on Ras pathway and suggest that they can influence the Ras activation state indirectly via regulators and effectors.

Key words: Redox; Guanine nucleotide exchange factor; GTPase activating protein; Raf; Reactive oxygen species; Signal transduction

1. Introduction

Reactive oxygen species (ROS) are responsible for a wide range of pathological processes such as DNA damage, oxidative inactivation of certain proteins and alteration of lipid molecular structure [1,2]. Free radicals are endogenously generated as normal by-products of the living cell and can be produced by various environmental chemicals. According to their broad action, in vivo experiments have shown that they can not only induce toxic effects but also play a crucial role in diverse physiological processes. In particular, the action of free radicals in vivo has been related to the specific activation of receptors, Ras pathway, mitogen-activated protein (MAP) kinase cascade, specific cyclins and the regulation of transcriptional and posttranscriptional events [3–5]. In this context, stimulation of various mammalian cell types with cytokines, phorbol esters or growth factors leads to the transient increase in the intracellular concentration of hydrogen peroxide, a response associated with tyrosine phosphorylation and MAP kinase stimulation [6–9]. Differently, fibroblasts transformed with an active form of Ras constitutively produce large amounts of ROS and exhibit a reduced tyrosine kinase activity in key elements of signalling, such as PDGF receptors and MAP kinases [10]. ROS generation has been shown to affect Ras signalling also in the yeast *Saccharomyces cerevisiae* [11]. On the basis of all these results it has been proposed that constitutive production of ROS in Ras-transformed cells activates intracellular pathways that are distinct from those activated by extracellular growth factors.

The mechanisms by which free radicals participate in all these processes remain in large part unknown. In fact, in contrast to the abundant literature dealing with effects of ROS in vivo, much less extended information exists on their direct targets and mode of action on purified components.

Since the investigation of the action of oxidizing agents in vitro has become a primary goal for understanding the molecular mechanism(s) by which the redox signalling is transmitted in the cell, in this work we have analyzed the action of the oxidizing agents H_2O_2, hemin and $HgCl_2$ on the activity of the Ras regulators GAP (GTPase activating protein) and GEF (GDP/GTP exchange factor) and on the binding between H-Ras and the downstream target c-Raf-1. Reversible inhibitory phenomena were found, whereas no effect was detected on the intrinsic activities of H-Ras or yeast *S. cerevisiae* Ras2p. These oxidizing agents appear to act on Ras proteins indirectly via regulators and effectors.

2. Materials and methods

2.1. Biological material

Purified H-Ras, Ras2p, the catalytic domain of the GEF $CDC25^{Mm}$ ($CDC25^{Mm285}$), p120-GAP (1042 amino acid residues), the catalytic domain of Ira2p-383 and the Ras binding domain of c-Raf-1 glutathione *S*-transferase (GST)-Raf (51–131) were obtained as reported [12,13].

2.2. GDP/GTP exchange activity

Dissociation of p21·nucleotide complexes was measured kinetically

*Corresponding author. E-mail: marco.vanoni@unimib.it

[1] Equivalent contributions were made by these two authors.
[2] Present address: Institut de Sciences Végétales, Unité Propre de Recherche no. 40, Centre National de la Recherche Scientifique, Bâtiment 23, Avenue de la Terrasse, F-91198 Gif-sur-Yvette Cedex, France.
[3] Also corresponding author. Fax: (33)-169333004; E-mail: andrea@pmc.polytechnique.fr.

Abbreviations: ROS, reactive oxygen species; GAP, GTPase activating protein; p120-GAP, human RasGAP; GEF, guanine nucleotide exchange factor; $CDC25^{Mm}$/GRF1, neuronal mouse RasGEF; GST, glutathione *S*-transferase; SPA, scintillation proximity assay; BSA, bovine serum albumin

0014-5793/01/$20.00
PII: S0014-5793(01)02251-7

at 30°C by nitrocellulose binding assay. The Ras·[^{3}H]GDP and Ras·[γ-^{32}P]GTP complexes (specific activity: 370 and 222 Bq/pmol, respectively) were obtained as described [13]. The reaction was started by addition of a 2000 times excess of cold GDP or GTP with or without GEF, H_2O_2, hemin or $HgCl_2$ (see legends). At time intervals, aliquots were passed on nitrocellulose filters (Sartorius SM 11 306, 0.45 μM) and the retained radioactivity measured in a liquid scintillator counter (Pharmacia, Wallac 1410).

2.3. GTPase activity

The GTPase activity was measured in the presence and in the absence of GAP proteins by nitrocellulose binding or the molybdate method. Ras·[γ-^{32}P]GTP complex (specific activity: 222 Bq/pmol) was obtained as in [14]. The reaction mixture contained H_2O_2, hemin or $HgCl_2$ (see legends). At time intervals, aliquots of the reaction mixture were passed through nitrocellulose filters and the retained radioactivity counted. The Ras·[γ-^{32}P]GTP hydrolysis was followed by determining the disappearance of radioactivity. With the molybdate method, Ras·[γ-^{32}P]GTP hydrolysis was determined via the release of $^{32}P_i$ as in [15]. The percentages of inhibition of Ras activities were always calculated in the linear portion of the time courses in the absence and in the presence of the indicated oxidizing agents.

2.4. Scintillation proximity assay (SPA)

The interaction between c-Raf-1 and H-Ras was monitored using SPA according to the procedure of Skinner et al. [16] and Gorman et al. [17] with the modifications described in [12]. After subtraction of the blanks, the SPA signal was expressed as a percentage of the Ras/GST-Raf binding relative to the control values obtained in the absence of H_2O_2, $HgCl_2$ or hemin. Since we have observed quenching phenomena between hemin and ^{3}H, 200 μM was the maximum final concentration of hemin used.

2.5. Growth of yeast cells and cyclic AMP (cAMP) determination

S. cerevisiae strain JM2763-14 was grown and treated with diethylmaleate (DEM) to induce mild oxidative stress as described [11]. Briefly, cells were grown to mid-exponential phase (ca. 5×10^6 cells/ml) in glucose-supplemented rich medium. Cells were then treated with 5, 10 and 15 mM DEM. After 60 and 120 min they were collected and processed for the determination of the levels of cAMP using the cAMP ^{3}H system from Amersham on control and DEM-treated cells, essentially as described [18].

3. Results

3.1. Oxidizing agents do not affect the intrinsic properties of Ras proteins

Intrinsic activities of H-Ras p21 have been reported to be influenced by nitric oxide, a powerful oxidizing compound produced in various metabolic processes of the cell [19–23]. In contrast to this neither H_2O_2 nor hemin even at high concentrations was able to affect the intrinsic GTPase of H-Ras p21 (Fig. 1A). Similarly, H_2O_2 and hemin did not alter the [^{3}H]GDP/GTP exchange of H-Ras p21 (Fig. 1B). In the case of $HgCl_2$ the abrupt dissociation of GDP from most H-Ras·GDP and the high concentration of $HgCl_2$ needed for this effect suggested the occurrence of unspecific denaturation phenomena as a major cause for this acceleration (Fig. 1C). In fact, comparable results to nucleotide dissociation were also found when the intrinsic GTPase activity of Ras was measured by filtration procedure, i.e. with a method determining the H-Ras-bound nucleotide, whereas with the molybdate method, that measures the GTP hydrolysis via liberation of $^{32}P_i$, this effect was not observed (Fig. 1D). The actions of oxidizing agents were also tested on the intrinsic dissociation

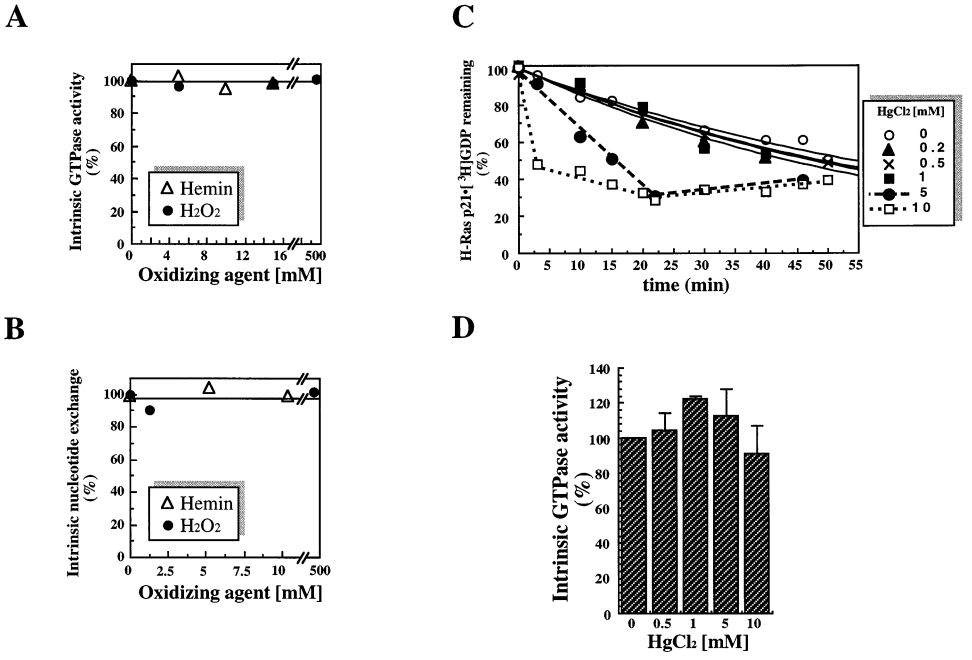

Fig. 1. Effect of oxidizing agents on the intrinsic properties of H-Ras. In A and B the intrinsic GTPase activity and nucleotide exchange of H-Ras were measured in 60 min kinetic experiments in the absence and the presence of increasing concentrations of hemin (△) or H_2O_2 (●), as described in Section 2. In A, the percentages of inhibition of intrinsic GTPase activity of H-Ras were determined versus the intrinsic GTPase activity of H-Ras in the absence of ROS that was taken as 100% activity. In B, the percentages of inhibition of intrinsic [^{3}H]GDP/GDP (●) or [^{32}P]GTP/GTP (△) exchange on H-Ras were determined versus the intrinsic nucleotide exchange on H-Ras in the absence of oxidizing agents, which was taken as 100% activity. In C, kinetics of dissociation of the p21·[^{3}H]GDP complexes were determined in the presence of increasing concentrations of $HgCl_2$. At the indicated time intervals, 15 μl aliquots were withdrawn and the amount of [^{3}H]GDP bound to H-Ras was determined by the nitrocellulose filtration procedure. In D, the intrinsic GTPase activity of H-Ras was measured in 50 min kinetic experiments in the presence of increasing concentrations of $HgCl_2$ by using the molybdate method as described in Section 2. The intrinsic GTPase activity on H-Ras in the absence of $HgCl_2$ was taken as 100% activity.

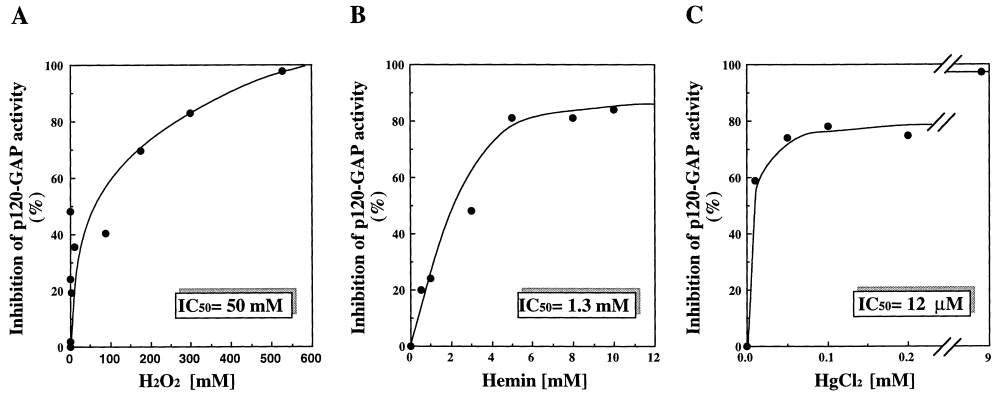

Fig. 2. Inhibition of GAP activity by oxidizing agents. 0.1 mM of H-Ras p21·[γ-^{32}P]GTP was added to a 100 μl mixture that contained fixed amounts of p120-GAP (●) (0.01–0.08 mM depending on the test) and the indicated increasing concentrations of H_2O_2 (A), hemin (B) or $HgCl_2$ (C) in buffer C (50 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, 1 mg/ml bovine serum albumin (BSA)). 10 μl aliquots were transferred to nitrocellulose disks at different intervals during 30 min kinetic experiments. The GTPase activity of p120-GAP was measured as the difference between the radioactivity retained in the absence and in the presence of the various agents. The GTPase activity in the absence of the agents was taken as 100%. The values are the average of several experiments, the standard deviations being $<15\%$.

rate of the *S. cerevisiae* Ras2p·GDP complex and the GTPase activity of Ras2p·GTP. Effects similar to those observed on H-Ras were obtained.

3.2. Inhibition of GAP activity

Due to its extremely low intrinsic GDP/GTP exchange and GTPase activity, the level of the active GTP-bound form of Ras in the cell depends on the ratio between the activities of the two regulators GEF and GAP [12]. Since no effect was observed on the intrinsic activities of Ras, we examined whether the regulators of Ras were susceptible of reversible oxidation regulating the activity of Ras pathway. As shown in Fig. 2A, high concentrations of H_2O_2 (IC_{50} in the millimolar range) inhibited human full-length p120-GAP with a dose-dependent effect. A more marked inhibition on GAP activity (Fig. 2B) was obtained with hemin; the IC_{50} was obtained at 1.35 mM hemin. $HgCl_2$ exerted an even stronger effect. In the range of 200–400 μM, it induced 100% inhibition of the p120-GAP activity (Fig. 2C). Similar effects were also obtained with yeast Ras2p by using the catalytic domain of the yeast GAP Ira2p-383 (Fig. 3).

The action on GAP of the three oxidizing agents was reversible. In the experiments shown in Fig. 5 (bottom panels), dilution of the reaction mixtures after incubation with the agent led to full or nearly full disappearance of the inhibition on GAP, whose stimulatory activity on H-Ras displayed values approaching those of the control.

3.3. Inhibition of the GEF-dependent nucleotide dissociation on Ras

The results described in the preceding paragraph establish for the first time a possible interference between Ras and its regulator GAP, dependent on oxidizing agents, under conditions where the intrinsic GTPase of Ras proteins was essentially unaffected. This prompted us to extend our analysis to GEF, the regulator regenerating the active form of Ras.

As for the full-length exchange factor $CDC25^{Mm}$ (not shown) and its catalytic domain ($CDC25^{Mm285}$), a situation comparable to that induced by GAP was observed. With H_2O_2 the IC_{50} was obtained at 10 mM (Fig. 4A). Hemin influenced negatively the activity of RasGEF $CDC25^{Mm285}$ more markedly than that of GAP, the IC_{50} being obtained at 40 μM hemin, while at 300–400 μM hemin the inhibition was 100% (Fig. 4B). The increase in concentration of $HgCl_2$ could inhibit nearly 100% the stimulation of the GDP/GTP activity of $CDC25^{Mm285}$ on H-Ras with an IC_{50} of 175 μM (Fig. 4C).

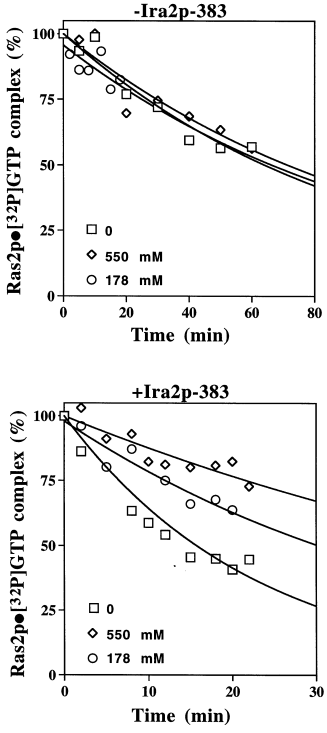

Fig. 3. Influence of H_2O_2 on Ira2p-383 activity. 0.1 mM of Ras2p·[γ-^{32}P]GTP was added to 100 μl of buffer C that contained increasing concentrations of H_2O_2 (0, 178, 550 mM) in the presence and in the absence of 55 nM of Ira2p-383. 10 μl aliquots were transferred to nitrocellulose disks at different intervals during 30 or 60 min kinetic experiments. The GTPase activity of Ira2p-383 was measured as the difference between the radioactivity retained in the absence and in the presence of Ira2p-383.

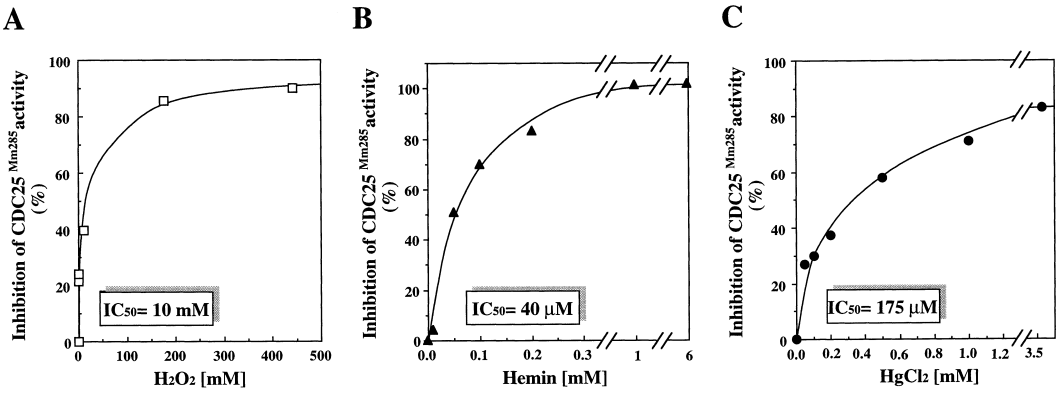

Fig. 4. Effect of oxidizing agents on the GEF-dependent GDP/GDP exchange on H-Ras. 0.1 μM of H-Ras p21·[^{3}H]GDP (A, C) or H-Ras p21·[γ-^{32}P]GTP (B) were added to a 100 μl mixture containing fixed amounts of CDC25^{Mm285} (0.01 μM) and the indicated concentrations of H_2O_2 (□), hemin (▲) or $HgCl_2$ (●) in buffer D (50 mM Tris–HCl, pH 7.5, 100 mM NH_4Cl, 1 mM $MgCl_2$, 1 mg/ml BSA). 10 μl aliquots were withdrawn and the amount of nucleotide-bound H-Ras was determined by nitrocellulose filtration procedure during 30 min kinetic experiments. The percentages of inhibition of CDC25^{Mm285} were determined taking the CDC25^{Mm285}-dependent p21 exchange activity in the absence of ROS as 100% activity. The values are the average of several experiments, the standard deviations being $<15\%$.

The inhibitory action of these agents was in large part reversible (Fig. 5), since after dilution of the reaction mixture the GEF activity approached that of the untreated control.

3.4. *Effect of oxidizing agents on the Ras–Raf interaction as monitored by SPA*

The SPA method allows the direct estimation of binding or inhibition of binding between two components without requiring a separation step, i.e. under equilibrium conditions of binding. This method, which uses fluoromicrospheres coated with anti-GST, allows the monitoring of the binding of GST-Raf-1 (51–131) to Ras p21 in complex with [^{3}H]GTP and the effect of oxidizing agents. As shown in Fig. 6A–C, all of the three agents inhibited the binding of c-Raf-1 to H-Ras. Hemin was most active, showing a maximum effect at 200 μM, at which concentration 80% of the reaction was inhibited. $HgCl_2$ was the second most powerful inhibitor, 90% inhibition of Raf binding to Ras being obtained in the range of 1–3 mM ($IC_{50} \sim 0.7$ mM). Finally, with H_2O_2, maximum inhibition (ca. 50%) was reached at a concentration around 10 mM.

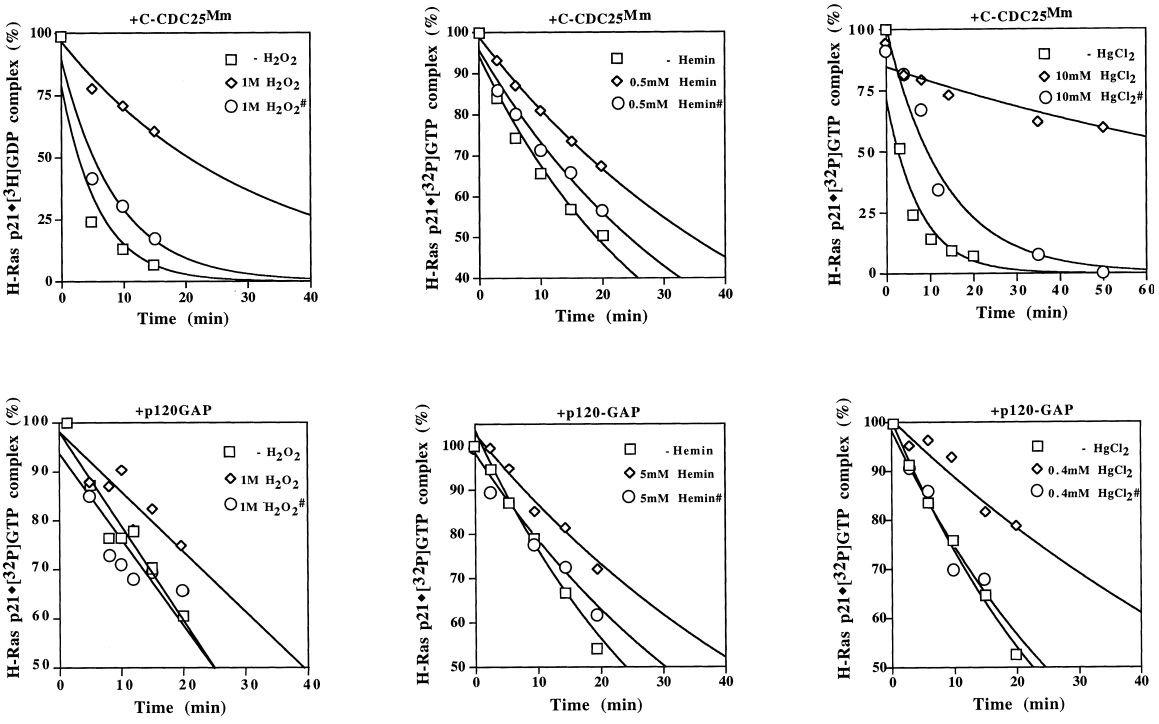

Fig. 5. The action of the agents on GAP and GEF activities is reversible. Experiments were performed as described in Figs. 2 and 3 and in Section 2. The symbol # indicates that the reaction mixtures, after incubation with the agent, were diluted about 500 times to get the same concentration of GAP and GEF and a lower concentration of the relative ROS.

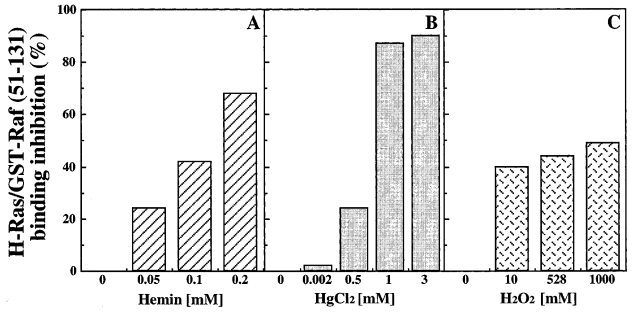

Fig. 6. The c-Raf (51–131) binding to H-Ras·[^{3}H]GTP is inhibited by hemin, $HgCl_2$ and H_2O_2. The indicated concentrations of hemin (A), $HgCl_2$ (B) or H_2O_2 (C) were added to SPA with 30 nM Ras·[^{3}H]GTP and 50 nM GST-Raf (51–131). After subtraction of the blanks without GST-Raf (51–131), the SPA signal was expressed as the percentage of inhibition relative to the control value obtained in the absence of oxidizing agents.

4. Discussion

Free radicals are important factors for the regulation of basic physiological processes and pathological events of the cell such as age-related alterations, atherosclerosis, carcinogenesis, diabetes and heat stress [1,2]. Most of the molecular phenotypes observed upon ROS increase are associated with a protective response of the cell against the possible toxic effects of these compounds [24]. More recently, a role for oxidizing agents in signal transduction has been proposed [5]. Depending on the cell type the biological effects of ROS can be dramatically different going from the activation of cell proliferation to induction of apoptosis and promotion of cell survival upon stress events [10,25–28]. The target(s) of these oxidizing species in the cell are not yet clearly defined, although experimental evidence suggests that they are located upstream/downstream of Ras and involve tyrosine phosphorylation [6,8,10]. Because of the importance of the upstream regulation of Ras, mediated by GAPs and GEFs and effectors (Raf-1, PI3-kinase and likely GAP) [12,14,29], we have analyzed whether their activities on Ras or in the case of Raf-1 the binding to H-Ras are affected by oxidizing agents. The oxidizing agents used in this work did not modify the intrinsic activities of Ras.

The oxidizing agents used influenced negatively the activity of GEF and GAP on H-Ras as well as the binding of c-Raf-1 to Ras. Since the intrinsic activities of H-Ras were not affected also at high concentrations of the oxidizing agents we could exclude that these inhibitions were due to denaturing phenomena of Ras. The extent of the inhibition greatly varied depending on the specific agent and Ras ligand. Noteworthy the negative effect of H_2O_2 cannot be directly quantified, since the action of H_2O_2 is mainly based on its very slow conversion to highly oxidizing derivatives, in particular the hydroxyl radical $^{\bullet}HO$. The H_2O_2 concentration modifying the action of GEF and GAP in our experiments was at least two orders of magnitude higher than the micromolar range of H_2O_2 estimated to be present in the cell [30–33]. This suggests a much higher sensitivity to H_2O_2 in vivo, probably depending on the involvement of other factors and metabolic steps, or on a selective subcellular localization of oxidizing agents. Hemin and $HgCl_2$ are more active inhibitors of the H-Ras regulators than H_2O_2. GAP is more sensitive to $HgCl_2$ than to hemin and H_2O_2, the negative effect being evident in the micromolar range, whereas GEF is more efficiently inhibited by hemin. The same results were observed using either mammalian or yeast Ras or when the full-length GAP or GEF or their catalytic domains were used. This suggests that the action of the oxidizing agents mainly concerns the catalytic regions of these Ras ligands. All the described effects were reversible, an important property because it grants to our in vitro observations a physiological relevance.

Table 1
Levels of cAMP (pmol/10^7 cells) in yeast cells treated with various DEM concentrations for various times

Time (min)	DEM concentration (mM)			
	0	5	10	15
0	0.50	–	–	–
60	0.49	0.21	0.26	0.31
120	0.46	1.02	0.12	0.90

For the assay conditions, see Section 2.

Oxidizing agents are able to modify any amino acid residue (for references: [2,34]); cysteine and methionine are particularly sensitive to their action, followed by histidine, arginine, lysine and proline. Aromatic amino acid residues are also preferred targets of ROS species. Protein fragmentation as the result of cleavage of peptide bond can follow the ROS attack on glutamyl, aspartyl and prolyl side chains. These more drastic effects can be ruled out, because of the reversibility observed under our experimental conditions. Recent studies have provided a structural basis of the molecular interaction between nitric oxide and H-Ras, pointing to the S-nitrosylation of cysteine 118 of H-Ras [20–22]. Our results show that H_2O_2, hemin and $HgCl_2$ have a comparable effect on both human H-Ras and *S. cerevisiae* Ras2p. Since the latter protein has a serine in position 125 as residue homologous to H-Ras cysteine 118, our observations decrease the relevance of the nature of this residue for oxidizing effects, at least for those induced by the agents used in this work. Noteworthy, the exposure of NIH3T3 cells to $HgCl_2$ was reported to induce both aggregation and activation of Src kinases as a consequence of the binding of two adjacent intermolecular SH groups forming a S–Hg–S bond [35–37].

Our results indicate that the negative effects of the three agents on Ras are mediated by upstream and downstream Ras ligands. Since it has been shown that Ras binding regions to GEF, GAP and Raf are partially overlapping and that these ligands compete for binding to Ras [12,14,38–40], our results suggest that ROS can affect the interactions between Ras and several of its ligands. The different potency of each oxidizing agent towards these ligands could induce either activation or inhibition of the Ras pathway, depending on whether either GAP or GEF is more severely affected. The stronger $HgCl_2$ inhibition of GAP would be expected to preferentially down-regulate GAP in vivo, thus mimicking GEF activation. Consistent with this possibility is the observation that the Ras/MAP kinase pathway in cultivated thymic lymphocytes is activated by $HgCl_2$ [41]. However, it is germane to mention that depending on the in vivo experimental situation, ROS generate different and sometimes opposite effects on mitogenic signals. In fact, ROS, generated constitutively in A6 cell lines transformed by overexpression of oncogenic H-Ras, can function differently [10] from the burst of ROS induced by

growth factor activating the Ras pathway [8]. The decreased activation of MAP kinase pathway observed in the former case is contrary to its activation by growth factor-induced ROS production or by the transient expression of oncogenic Ras in non-transformed cells [8,9]. Moreover, although an increased tyrosine kinase activity has been shown to occur after addition of exogenous ROS in various cell lines including NIH3T3 [37,42–44], the production of ROS in Ras-transformed fibroblasts is accompanied by a reduction of tyrosine kinase activity of different key signalling molecules. To explain this discrepancy, it has been proposed that constitutive production of ROS in Ras-transformed A6 cells activates intracellular pathways that may be distinct from those activated by extracellular growth factors [10]. Our in vitro observations that oxidizing agents induce a direct inhibition of the binding of H-Ras to c-Raf-1, the first element of the MAP kinase cascade, correspond to the situation in which ROS are constitutively produced. This suggests that in vivo the activation by ROS of the MAP pathway is dependent on elements other than Ras. Even though it was initially thought that Ras plays the major role in the activation of MAP kinases, activation of this pathway has later been shown to be mediated also by other signalling molecules such as Rap1, PKC, Rac1 and Cdc42 [45–48]. Accordingly, experiments in vivo have supported an important involvement of the Rac pathway in the action of ROS [49–51]. H_2O_2 has been shown to activate PKC isoforms influencing the activity of components of the MAP kinase pathway [28].

To further prove that our in vitro results are of physiological relevance, we re-investigated the effect of mild oxidative stress on the Ras pathway in *S. cerevisiae* in vivo. We previously showed that treatment with low doses of DEM, a glutathione depleter, down-regulates the Ras pathway as shown by a G0-like cell cycle arrest and induction of a STRE-driven reporter gene [11]. Since these are all integrative long-term effects, we decided to directly measure the intracellular levels of cAMP, i.e. the immediate downstream target of Ras in budding yeast. Exponentially growing yeast cells were treated with 5, 10 or 15 mM DEM. Samples were taken at different times after DEM treatment and processed for determination of cAMP levels. Results are reported in Table 1 and indicate that 1 h after DEM addition, a strong reduction in cAMP level is observed (Table 1). These results are consistent with the reported effects on glutathione levels and expression of reporter genes [11]. One hour later, however, some feedback mechanism counteracting the DEM effect becomes operative, so that the cAMP returns at levels even higher than that of control cells. These observations indicate that the Ras/cAMP/PKA pathway is a major target of ROS action in yeast, but that it is affected only transiently. Later on, a second Ras-independent pathway takes over, resulting in cell growth arrest. Consistently, hyperactivation of the Ras pathway (brought about by either overexpression of the yeast GEF CDC25 or by expression of the activated $Ras2^{Val19}$ protein) rescues the DEM-induced G1 arrest, but fails to rescue the growth defect of DEM-treated cells that arrest as budded G2/M cells [11]. Thus the above data indicate that proteins involved in regulation of the Ras cycle may be direct targets of oxidative stress in eukaryotes. Notably, recent evidence has shown that not only the small G proteins, but the α subunits of trimeric G proteins, $G\alpha_i$ and $G\alpha_0$, can also be the direct target for ROS action [52].

In conclusion, this work shows that the upstream regulators of Ras, as well as its downstream effectors, represent additional potential targets for the action of ROS. The intrinsic function of Ras is not directly involved but it can be strongly influenced via the response of regulators and effectors to oxidizing compounds. The activity of GAP and GEF is known to control the level of the active form of Ras and very likely also Ras effectors such as Raf-1 and PI3-kinase can indirectly participate with a kind of feedback mechanism to the regulation of the level of the active Ras·GTP complex by modulating the activity of GEF [12,14]. As a consequence, the action of ROS on the Ras pathway very probably takes place via indirect tuning of the switch functions of Ras.

Acknowledgements: We thank our laboratory colleagues Drs. S. Baouz and E. Jacquet for providing purified $CDC25^{Mm}$ and C-$CDC25^{Mm285}$ and S. Busti for help with the cAMP assays. This work was supported by the EC contract BIOTECH BIO4-CT96-1110, Ligue Nationale Française Contre le Cancer, Association pour la Recherche sur le Cancer (Grant no. 9846), Consiglio Nazionale delle Ricerche 'Target Project on Biotechnology' and MURST-CNR Biotechnology Program L. 95/95.

References

[1] Yu, B.P. (1994) Physiol. Rev. 74, 139–162.
[2] Berlett, B.S. and Stadtman, E.R. (1997) J. Biol. Chem. 272, 20313–20316.
[3] Cimino, F., Esposito, F., Ammendola, R. and Russo, T. (1996) Curr. Top. Cell Regul. 35, 123–148.
[4] Kamata, H. and Hirata, H. (1999) Cell Signal. 11, 1–14.
[5] Pennesi, E. (1997) Science 275, 1567–1568.
[6] Shibanuma, M., Kuroki, T. and Nose, K. (1990) Oncogene 5, 1025–1032.
[7] Ohba, M., Shibanuma, M., Kuroki, T. and Nose, K. (1994) J. Cell. Biol. 4, 1079–1088.
[8] Sundaresan, M., Yu, Z.X., Ferrans, V.J., Irani, K. and Finkel, T. (1995) Science 270, 296–299.
[9] Lo, Y.Y. and Cruz, T.F. (1995) J. Biol. Chem. 270, 11727–11730.
[10] Irani, K., Xia, Y., Zweier, J.L., Sollott, S.J., Der, C.J., Fearon, E.R., Sundaresan, M., Finkel, T. and Goldschmidt-Clermont, P.J. (1997) Science 275, 1649–1652.
[11] Wanke, V., Accorsi, K., Porro, D., Esposito, E., Russo, T. and Vanoni, M. (1999) Mol. Microbiol. 32, 753–764.
[12] Giglione, C. and Parmeggiani, A. (1998) J. Biol. Chem. 273, 34737–34744.
[13] Parrini, M.C., Jacquet, E., Bernardi, A., Jacquet, M. and Parmeggiani, A. (1995) Biochemistry 34, 13776–13783.
[14] Giglione, C., Parrini, M.C., Baouz, S., Bernardi, A. and Parmeggiani, A. (1997) J. Biol. Chem. 272, 25128–25134.
[15] Parmeggiani, A., Singer, C. and Gottschalk, E.M. (1971) Methods Enzymol. 20, 291–302.
[16] Skinner, R.H., Picardo, M., Gane, N.M., Cook, N.D., Morgan, L., Rowedder, J. and Lowe, P.N. (1994) Anal. Biochem. 223, 259–265.
[17] Gorman, C., Skinner, R.H., Skelly, J.V., Neidle, S. and Lowe, P.N. (1996) J. Biol. Chem. 271, 6713–6719.
[18] Thevelein, J.M., Beullens, M., Honshoven, F., Hoebeeck, G., Detremerie, K., Griewel, B., den Hollander, J.A. and Jans, A.W.H. (1987) J. Gen. Microbiol. 133, 2197–2205.
[19] Lander, H.M., Ogiste, J.S., Frieda, S.A., Levi, R. and Novogrodsky, A. (1995) J. Biol. Chem. 270, 7017–7020.
[20] Lander, H.M., Milbank, A.J., Tauras, J.M., Hajjar, D.P., Hempstead, B.L., Schwartz, G.D., Kraemer, R.T., Mirza, U.A., Chait, B.T., Burk, S.C. and Quilliam, L.A. (1996) Nature 381, 380–381.
[21] Lander, H.M., Hajjar, D.P., Hempstead, B.L., Mirza, U.A., Chait, B.T., Campbell, S.L. and Quilliam, L.A. (1997) J. Biol. Chem. 272, 4323–4326.
[22] Mott, H.R., Carpenter, J.W. and Campbell, S.L. (1997) Biochemistry 36, 3640–3644.
[23] Lander, H.M., Ogiste, J.S., Teng, K.K. and Novogrodsky, A. (1995) J. Biol. Chem. 270, 21195–21198.

[24] Halliwell, B. and Gutteridge, J.M.C. (1986) Arch. Biochem. Biophys. 246, 501–514.
[25] Hockenbery, D.M., Oltvai, Z.N., Yin, X.M., Milliman, C.L. and Korsmeyer, S.J. (1993) Cell 75, 241–251.
[26] Kane, D.J., Sarafian, T.A., Anton, R., Hahn, H., Gralla, E.B., Valentine, J.S., Ord, T. and Bredesen, D.E. (1993) Science 162, 1274–1277.
[27] Haecker, G. and Vaux, D.J. (1994) Trends Biochem. Sci. 19, 99–100.
[28] Guyton, K.Z., Liu, Y., Gorospe, M., Xu, Q. and Holbrook, N.J. (1996) J. Biol. Chem. 271, 4138–4142.
[29] McCormick, F. and Wittinghofer, A. (1996) Curr. Opin. Biotechnol. 7, 449–456.
[30] Chance, B., Sies, H. and Boveris, A. (1979) Physiol. Rev. 59, 527–605.
[31] Beckman, J.S. (1994) in: The Neurobiology of NO and OH (Chiueh, C.C., Gilbert, D.L. and Colton, C., Eds.), Ann. N.Y. Acad. Sci. 738, 69–75.
[32] Malinsky, T., Bailey, F., Zhang, Z.G. and Chopp, M. (1993) J. Cereb. Blood Flow Metab. 13, 355–358.
[33] Morel, Y. and Barouki, R. (1999) Biochem. J. 342, 481–496.
[34] Stamler, J.S. and Hausladen, A. (1998) Nat. Struct. Biol. 5, 247–249.
[35] Sperling, R., Burstein, Y. and Steinberg, I.Z. (1969) Biochemistry 8, 3810–3820.
[36] Steer, M.L., Tal, N. and Lewitzki, A. (1974) Biochim. Biophys. Acta 334, 389–397.
[37] Pu, M.Y., Akhand, A.A., Kato, M., Koike, T., Hamaguchi, M., Suzuki, H. and Nakashima, I. (1996) J. Cell. Biochem. 63, 104–114.
[38] Boriack-Sjodin, P.A., Margarit, S.M., Bar-Sagi, D. and Kuriyan, J. (1998) Nature 394, 337–343.
[39] Sheffzek, K., Ahmadian, M.R., Kabsch, W., Wiesmüller, L., Lautwein, A., Schmitz, F. and Wittinghofer, A.Z. (1997) Science 277, 333–338.
[40] Nassar, N., Horn, G., Herrmann, C., Scherer, A., McCormick, F. and Wittinghofer, A. (1995) Nature 375, 554–560.
[41] Akhand, A.A., Kato, M., Suzuki, H., Miyata, T. and Nakashima, I. (1998) J. Cell Biochem. 71, 243–560.
[42] Devary, Y., Gottliebn, R.A., Smeal, T. and Karin, M. (1992) Cell 71, 1081–1091.
[43] Nakamura, K., Hori, T., Sato, N., Sugie, K., Kawakami, T. and Yodoi, J. (1993) Oncogene 11, 3133–3139.
[44] Hardwick, J.S. and Sefton, B.M. (1995) Proc. Natl. Acad. Sci. USA 92, 4527–4531.
[45] Lolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., Marmé, D. and Rapp, U.R. (1993) Nature 364, 249–252.
[46] Carroll, M.P. and May, S. (1994) J. Biol. Chem. 269, 1249–1256.
[47] Ueda, Y., Hirai, S., Osada, A., Suzuki, K., Mizuno, K. and Ohno, S. (1996) J. Biol. Chem. 271, 23512–23519.
[48] Schaeffer, H.J. and Weber, M.J. (1999) Mol. Cell. Biol. 19, 2435–2444.
[49] Sundaresan, M., Yu, Z.X., Ferrans, V.J., Sulciner, D.J., Gutkind, J.S., Irani, K. and Goldschmidt-Clermont, P.J. (1996) Biochem. J. 1, 379–382.
[50] Sulciner, D.J., Irani, K., Yu, Z.X., Ferrans, V.J., Goldschmidt-Clermont, P.J. and Finkel, T. (1996) Mol. Cell. Biol. 16, 7115–7121.
[51] Cool, R.H., Merten, E., Theiss, C. and Acker, H. (1998) Biochem. J. 15, 5–8.
[52] Nishida, M., Maruyama, Y., Tanaka, R., Kontani, K., Nagao, T. and Kurose, H.G. (2000) Nature 408, 492–495.