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Superoxide Anion Radical Modulates the Activity of Ras and Ras-related GTPases by a Radical-based Mechanism Similar to That of Nitric Oxide*

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Ras GTPases cycle between inactive GDP-bound and active GTP-bound states to modulate a diverse array of processes involved in cellular growth control. The activity of Ras is up-regulated by cellular agents, including both protein (guanine nucleotide exchange factors) and redox-active agents (nitric oxide (NO) and superoxide anion radical ($O_2^{\cdot-}$)). We have recently elucidated the mechanism by which NO promotes guanine nucleotide dissociation of redox-active NKCD motif-containing Ras and Ras-related GTPases. In this study, we show that guanine nucleotide dissociation is enhanced upon exposure of the redox-active GTPases, Ras and Rap1A, to $O_2^{\cdot-}$ and provide evidence for the efficient guanine nucleotide reassociation in the presence of the radical quenching agent ascorbate to complete guanine nucleotide exchange. *In vivo*, guanine nucleotide reassociation is necessary to populate Ras in its biologically active GTP-bound form after the dissociation of GDP. We further show that treatment of the redox-active GTPases with $O_2^{\cdot-}$ releases GDP in the form of an unstable oxygenated GDP adduct, putatively assigned as 5-oxo-GDP. 5-Oxo-GDP was not produced from either the C118S or the F28L Ras variants upon the treatment of $O_2^{\cdot-}$, supporting the involvement of residues Cys^{118} and Phe^{28} in $O_2^{\cdot-}$-mediated Ras guanine nucleotide dissociation. These results indicate that the mechanism of $O_2^{\cdot-}$-mediated Ras guanine nucleotide dissociation is similar to that of NO/O_2-mediated Ras guanine nucleotide dissociation.

The $p21_{Ras}$ proto-oncoprotein (Ras) is a founding member of the Ras superfamily of GTPases and plays an important role in a number of biological processes involved in cellular growth control, including cell growth, differentiation, and apoptosis (1, 2). Ras cycles between an inactive GDP-bound and active GTP-bound form to modulate association with regulators and downstream targets. High affinity interactions with downstream effectors are achieved via interaction with GTP-bound Ras rather than the GDP-bound form of Ras, which in turn leads to effector activation, stimulation of downstream signaling pathways, and a plethora of biological responses (3–5). Given the importance of Ras in various growth control processes, the population of Ras in its biologically active GTP-bound or inactive GDP-bound form is critically regulated by cellular factors. For example, the intrinsically slow rate of Ras guanine nucleotide dissociation and GTP hydrolysis is modulated by regulatory proteins and free radicals. GTPase-activating proteins down-regulate Ras activity by stimulating the intrinsically slow rate of GTP hydrolysis to populate Ras in its inactive GDP-bound form, whereas guanine nucleotide exchange factors, reactive nitrogen species, and reactive oxygen species (ROS)[1] (6–18) up-regulate Ras function by promoting guanine nucleotide exchange (GNE) to generate the active GTP-bound state of Ras *in vivo*. A number of reactive species may contribute to the regulation of Ras activity, including the reactive nitrogen species nitric oxide (NO), nitrogen dioxide ($^{\cdot}NO_2$), and dinitrogen trioxide (N_2O_3) as well as ROS such as the superoxide anion radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2).

NO is the best characterized redox modulator of Ras activity as NO has been shown to promote Ras GDP dissociation *in vitro*, GTP binding to Ras *in vivo*, and stimulation of pathways downstream of Ras (9–14, 19, 20). The target site of NO modification and NO-mediated guanine nucleotide dissociation on Ras is Cys^{118}, which is located in the nucleotide-binding NKCD motif (11, 12, 20, 21). Our recent studies indicated that $^{\cdot}NO_2$, a reaction product of NO with O_2, reacts with the Ras Cys^{118} thiol to produce a Ras Cys^{118}-thiyl radical (Ras-$S^{118\cdot}$) intermediate (22). Once generated, Ras-$S^{118\cdot}$ induces a series of radical reactions involving the Phe^{28} side chain and the Ras-bound GDP base to cause the conversion of Ras-bound GDP into a Ras-bound GDP neutral radical ($G^{\cdot}$-DP) (23). Ras-bound $G^{\cdot}$-DP further reacts with an additional $^{\cdot}NO_2$ to produce a GDP-NO_2 adduct, 5-nitro-GDP. 5-Nitro-GDP can then be degraded into NIm-DP by decarboxylation of the 5-nitro-GDP C_6 atom. This NO/O_2-mediated radical-based process perturbs Ras GDP binding interactions, resulting in the dissociation of GDP from Ras in the form of 5-nitro-GDP (23).

In addition to NO, ROS have also been shown to activate GNE on Ras and Ras-related GTPases (8, 15). Early evidence for ROS-mediated activation of Ras was obtained in studies in which various oxidative agents (*i.e.* H_2O_2 and hemin) could enhance GNE on Ras *in vitro* and enhance Ras-mediated mitogen-activated protein (MAP) kinase activity in Jurkat cells

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[1] The abbreviations used are: ROS, reactive oxygen species; NO, nitric oxide; WT, wild type; GNE, guanine nucleotide exchange; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; AIm-DP, 5-amino-4*O*-imidazolone ribose diphosphate; AOn-DP, 5-amino-oxazolone ribose diphosphate; DIm-DP, 5-diamino-4*O*-imidazolone ribose diphosphate; IIm-DP, 5-imino-4*O*-imidazolone ribose diphosphate; NIm-DP, 5-guanidino-4-nitroimidazole; Mant, *N*-methylanthraniloyl.

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(15). Moreover, activation of NF-κB activity by these oxidative agents was blocked by expression of a dominant negative Ras mutant, suggesting that direct activation of Ras may be a central mechanism by which a variety of oxidative redox-stress stimuli transmit their signals to the nucleus (15). Recently, Adachi *et al.* (8) showed that H_2O_2-induced modification of Ras at Cys^{118} facilitates activation of p38 and Akt but not extracellular signal-regulated kinase (ERK) activation in rat vascular smooth muscle cells.

The enzymes NADPH oxidase (24), xanthine oxidase (25–27), and nitric oxide synthase (28–33) generate the ROS, $O_2^{\cdot-}$. Notably, the redox potential of $O_2^{\cdot-}$ (E_7 = ~0.9 *versus* normal hydrogen electrode (NHE)) is higher than that of thiyl radical (R-S$^\cdot$) (E_7 = ~0.8 V *versus* NHE) (34–36). Therefore, like $^\cdot NO_2$, $O_2^{\cdot-}$ may serve as a redox-signaling agent for NKCD motif-containing Ras and Ras-related GTPases. Results obtained from several *in vivo* studies (16–18, 37–39) lend support to this hypothesis. For example, a constitutively active oncogenic Ras variant was observed to up-regulate expression of the NADPH homologue, Nox1 (17, 39) via the MAP kinase pathway (16). Conversely, this study also showed that small interfering RNA-mediated inhibition of Nox1 expression blocked transforming phenotypes (anchorage-independent growth, morphological changes, and production of tumors in athymic mice) associated with Ras up-regulation (16), suggesting that the production of $O_2^{\cdot-}$ by Nox1 is required for oncogenic Ras transformation. Similarly, it has been shown that Ras-triggered cell growth can be blocked by suppression of $O_2^{\cdot-}$ production from NADPH oxidase (18). Intriguingly, the redox-active NKCD motif-containing Rap1A, a member of the Ras subclass of GTPases, has been observed to colocalize with NADPH oxidase (40). Although Ras and Rap proteins commonly regulate activation of the MAP kinase cascade in some cell types (41), they regulate distinct cellular effectors. In fact, Rap GTPases are best characterized as critical regulators of integrin-mediated cell adhesion; however, their mechanism of action is poorly understood (42, 43). Since the redox-active Rap1A GTPase colocalizes with NADPH oxidase (40) and is sensitive to NO (19, 20), it is possible that Rap1A activity may also be regulated by $O_2^{\cdot-}$ produced from NADPH oxidase. Although these *in vivo* results support the intriguing possibility that $O_2^{\cdot-}$ mediates regulation of Ras and Ras-related GTPase activities, direct modulation has not been demonstrated.

Results from this study demonstrate that $O_2^{\cdot-}$ facilitates guanine nucleotide dissociation from Ras as well as Rap1A. We also provide evidence that the molecular mechanism of $O_2^{\cdot-}$-mediated guanine nucleotide dissociation is similar to that of the NO/O_2-mediated guanine nucleotide dissociation.

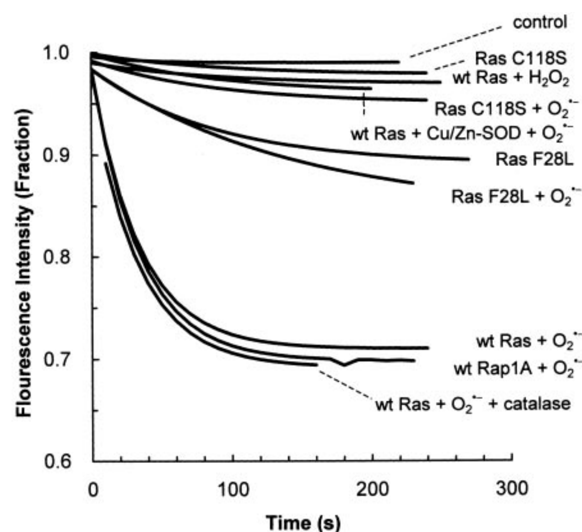

FIG. 1. **Fluorescence-based determination of $O_2^{\cdot-}$-mediated guanine nucleotide dissociation from WT Ras, Ras variants, and WT Rap1A.** Prior to initiating the fluorescence assay, both $^{XO\ pseudo}k$ and [$O_2^{\cdot-}$], to be generated during the assay, were quantified for the assay time using a methylene blue-coupled assay (see details under "Experimental Procedures"). Fluorescence mant-GDP-loaded Ras or Rap1A (0.5 μM) and xanthine oxidase (activity: 0.5 μM [$O_2^{\cdot-}$] s^{-1}) in the absence and presence of catalase (~1,000 units) were transferred into sealed assay cuvettes containing assay buffer at pH 7.5, and the decrease in fluorescence emission at 460 nm was recorded as a function of time (300 s). Apparent $O_2^{\cdot-}$-mediated mant-GDP dissociation rates of 0.02×10^{-3}, 1.16×10^{-3}, 2.21×10^{-3}, and 2.18×10^{-3} M s^{-1} for Ras F28L, WT Ras, WT Ras with catalase, and WT Rap1A, respectively, were determined by fitting the data to a simple exponential decay. The estimated apparent rates were converted into true $O_2^{\cdot-}$-mediated mant-GDP dissociation rates of 3.11×10^{-3} and 3.12×10^{-3} s^{-1} for WT Ras and WT Rap1A, respectively, according to Equation 2. For comparison, the intrinsic rates of GDP dissociation rates for WT Ras and the C118S and F28L variants were also measured. Rates of apparent intrinsic GDP dissociation (in the absence of $O_2^{\cdot-}$) corresponding to 0.05×10^{-3}, 1.39×10^{-3}, 0.02×10^{-3}, and 0.02×10^{-3} s^{-1} for Ras C118S, Ras F28L, WT Ras, and WT Rap1A, respectively, were determined by fitting the data to a simple exponential decay. As a control, Cu,Zn-superoxide dismutase was employed to remove $O_2^{\cdot-}$ from the reaction mixture. Cu,Zn-superoxide dismutase (~5,000 units) was introduced before the addition of WT Ras. The rate of apparent $O_2^{\cdot-}$-mediated WT Ras GDP dissociation in the presence of Cu,Zn-superoxide dismutase was determined to be 0.04×10^{-3} s^{-1}, which is similar to the intrinsic rate of Ras GDP dissociation. We also assessed whether H_2O_2 mediates WT Ras guanine nucleotide dissociation. Identical experimental conditions for $O_2^{\cdot-}$-mediated Ras guanine nucleotide dissociation were employed except that H_2O_2 (1%) was used instead of xanthine oxidase in the assay system. The rate of H_2O_2-mediated mant-GDP dissociation was determined by fitting the data to a simple exponential decay to give 0.07×10^{-3} s^{-1}. For all results, regression values of $r^2 > 0.9505$ were obtained with a standard error of <8%.

EXPERIMENTAL PROCEDURES

Preparation of Chemicals—The chemicals used for all experiments were of the highest grade unless otherwise noted. Radiolabeled GDP ([^{3}H]GDP) was obtained from PerkinElmer Life Sciences. The radiolabeled GDP was diluted with unlabeled guanine nucleotide prior to use, giving ~1,000 dpm/μM GDP. Uniformly enriched [^{13}C-^{15}N]GDP (95%) was obtained from Martek.

Preparation of Protein Samples—Human H-Ras-(1–166), Ras F28L, Ras C118S, and Rap1A-(1–184) were expressed and purified as described previously (44, 45). The final proteins were >95% pure as determined by SDS-PAGE. The protein concentration was determined by the Bradford method (46). Xanthine oxidase was purchased from Sigma. Bovine liver catalase (40,000 units/mg) and bovine liver superoxide dismutase (50,000 units/mg, Cu,Zn-superoxide dismutase) were purchased from Sigma and further purified using a size-exclusion column (Sephacryl S-100, 2.5 × 60 cm) prior to use.

Generation and Quantification of $O_2^{\cdot-}$—Xanthine oxidase was used to generate $O_2^{\cdot-}$. All kinetic assays and mass spectrometry (MS) sample preparations were performed in serum-stoppered sealed cuvettes and vials to avoid diffusion of xanthine oxidase produced $O_2^{\cdot-}$ into the open atmosphere. To prevent the transition metal-mediated conversion of the $O_2^{\cdot-}$ radical into H_2O_2 and OH$^\cdot$ (47), all buffers used for kinetic and biochemical assays were passed through a metal-chelating Bio-Rad Chelex-100 cation exchange column (48), and EDTA (0.5 mM) was added to the buffers prior to performing the experiments, unless otherwise noted.

The rate of $O_2^{\cdot-}$ production by xanthine oxidase (^{XO}k) was determined by employing a methylene blue-coupled assay. Briefly, various amounts of xanthine oxidase were added to assay buffer containing 0.5 μM oxidized methylene blue, 10 mM xanthine, 20 mM GDP, 50 mM NaCl, 5 mM $MgCl_2$, and 0.5 mM EDTA in ammonium acetate buffer (10 mM, pH 7.5). The decrease in absorbance at 500 nm monitored over time (0–10 s) using a Shimadzu UV-2501 spectrophotometer corresponds to reduction of oxidized methylene blue due to coupling with xanthine oxidase-mediated oxidation of xanthine to uric acid. It has been reported that xanthine oxidase produces ~30% $O_2^{\cdot-}$ and ~70% H_2O_2/enzyme turnover in the presence of its substrates O_2 and xanthine (49). Given these values (49), the total amount of $O_2^{\cdot-}$ produced by xanthine oxidase for a given time *t* can be approximated following Equation 1.

$$[O_2^{\cdot-}] \approx 0.3 \times {}^{XO}k \times t \quad \text{(Eq. 1)}$$

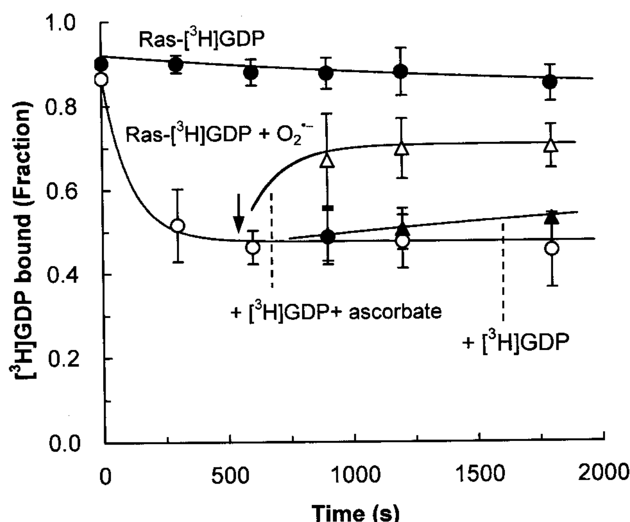

FIG. 2. **Determination of $O_2^{\cdot-}$-mediated Ras guanine nucleotide GNE employing ^{3}H-labeled GDP.** Xanthine oxidase was employed to produce $O_2^{\cdot-}$ similar to that described in the legend for Fig. 1. The assay mixture used to monitor Ras GDP dissociation contained 10 mM xanthine, 20 mM GDP, 50 mM NaCl, 5 mM $MgCl_2$, and 0.5 mM EDTA in ammonium acetate buffer (10 mM, pH 7.5). [^{3}H]GDP-loaded Ras (2 μM) was added to the assay solution followed by the addition of xanthine oxidase. An excess of [^{3}H]GDP (100 mM) in the presence and absence of the radical quencher, ascorbate (1 mM), was additionally introduced to the assay mixture at the assay time 500 s (indicated by the *arrow*). As a control experiment, the rate of intrinsic Ras GDP dissociation was also measured using experimental conditions identical to those described above, except that xanthine oxidase was not added. For all assays, aliquots were withdrawn at specific time points and spotted onto nitrocellulose filters. The filters were then washed three times with assay buffer, and radioactivity was determined using Beckman-Coulter scintillation counter. The resultant radioactivity (dpm values) associated with Ras-bound [^{3}H]GDP was converted into the fraction of mol of GDP/mol of total Ras. The apparent WT Ras GDP dissociation rates in the absence and presence of $O_2^{\cdot-}$ were determined to be 0.02×10^{-3} and 1.89×10^{-3} M s^{-1}, respectively, by fitting the data to a simple exponential decay. The estimated apparent Ras GDP dissociation rate in the presence of $O_2^{\cdot-}$ was converted into true a $O_2^{\cdot-}$-mediated WT Ras GDP dissociation (3.9×10^{-3} s^{-1}), according to Equation 2. The apparent WT Ras GDP association rates in the absence and presence of ascorbate were determined to be 0.14×10^3 and 1.61×10^3 s^{-1}, respectively, by fitting the data to a simple exponential association. Since the association process observed in these assays could include the sequential process of Ras GDP dissociation followed by Ras GDP association, the conversion of the apparent WT Ras GDP association rates into the corresponding true values could not be easily conducted. Hence, the estimated values for the apparent WT Ras GDP association rates were not converted into true kinetic values. The data presented in this figure are mean values of triplicate measurements. Standard errors of each data point are <48%, and the regression values associated with the fit were $r^2 > 0.7595$. The β-errors associated with entire assay process were not included to account for the standard errors and r^2 values.

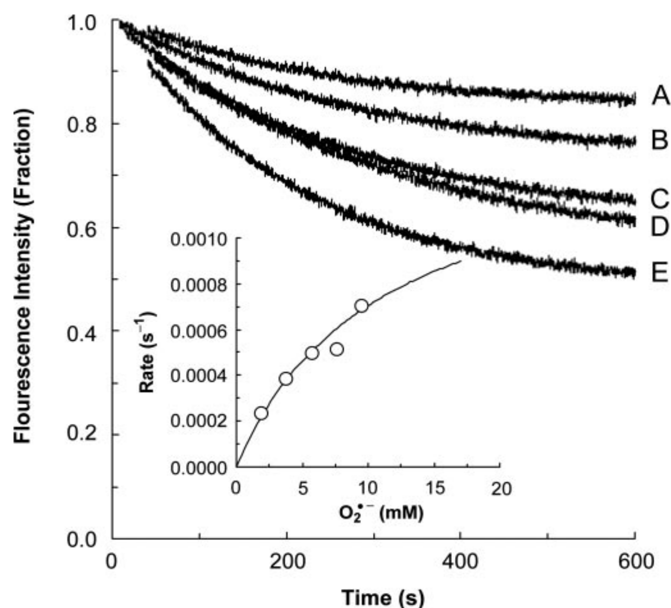

FIG. 3. **Determination of the Ras GDP dissociation constant in the presence of $O_2^{\cdot-}$.** The activity of xanthine oxidase activity (5.1 μM^{-1} s^{-1}) used in this assay was determined using a methylene blue-coupled assay. $O_2^{\cdot-}$-producing xanthine oxidase was incubated in sealed assay cuvettes containing assay buffer at pH 7.5 for 10 (*A*), 20 (*B*), 30 (*C*), 40 (*D*), and 50 (*E*) min prior to the addition of mant-GDP-loaded Ras. Estimated $O_2^{\cdot-}$ concentrations of ~1.9, 3.8, 5.7, 7.6, and 9.5 mM were generated in the assay using Equation 1. Fluorescence mant-GDP-loaded Ras (0.5 μM) was added to $O_2^{\cdot-}$-containing assay cuvettes, and the decrease in fluorescence emission at 460 nm was recorded as a function of time. The initial $O_2^{\cdot-}$-mediated Ras GDP dissociation rates were determined over 0–10 s and fit to a simple exponential decay. Regression values of the fit were $r^2 > 0.9895$. The estimated values were then plotted against the concentration of $O_2^{\cdot-}$, and ${}^{O_2^{\cdot-}}K_D$ (11.3 mM) was determined by fitting the plot to a simple hyperbola (*inset*), where the regression value of the fit was $r^2 = 0.8795$. Using the ${}^{O_2^{\cdot-}}K_D$ and ${}^{\text{true } O_2^{\cdot-}}k_{\text{off}}$ (Fig. 1) values determined according to Equation 3, the true rate of mant-GDP association (${}^{\text{true } O_2^{\cdot-}}k_{\text{on}} = 0.27$ M^{-1} s^{-1}) was determined.

Kinetic Measurements of Guanine Nucleotide Exchange on Ras and Rap1A—Excess amounts of xanthine oxidase, its substrate xanthine, or catalase do not interfere with either the fluorescence intensity of the mant-labeled 2′-(or-3′)-*O*-(*N*-methylanthraniloyl)guanosine 5′-diphosphate (mant-GDP) or the GDP binding properties of Ras and Rap1A. Therefore, we employed a mant-GDP fluorescence assay (50, 51) to conduct kinetics studies of $O_2^{\cdot-}$-mediated Ras and Rap1A guanine nucleotide dissociation. Wild-type (WT) Ras, the Ras variants C118S and F28L, and Rap1A were preloaded with mant-GDP as described previously (50, 51). A standard assay mixture for the fluorescence measurements consisted of 10 mM xanthine, 20 mM GDP, 50 mM NaCl, 5 mM $MgCl_2$, and 0.5 mM EDTA in ammonium acetate buffer (10 mM, pH 7.5). Once the GTPases was loaded with mant-GDP, xanthine oxidase was added, and the dissociation of mant-GDP from the GTPase was measured as a change in the fluorescence intensity over time using a LS50B PerkinElmer Life Sciences fluorimeter. Apparent second-order rates of $O_2^{\cdot-}$-mediated Ras and Rap1A GDP dissociation (${}^{\text{app } O_2^{\cdot-}}k_{\text{off}}$) were determined by fitting the data to a simple exponential decay.

We also employed ^{3}H-radiolabeled GDP to assess the rates of Ras GDP dissociation and association in the presence of $O_2^{\cdot-}$. To measure the rate of $O_2^{\cdot-}$-mediated Ras GDP dissociation, [^{3}H]GDP-preloaded Ras was treated with $O_2^{\cdot-}$ in the presence of free unlabeled GDP, and the dissociation of [^{3}H]GDP from Ras was measured as a change in Ras radioactivity over time using Beckman-Coulter scintillation counter. To assess the rate of $O_2^{\cdot-}$-mediated Ras GDP association in the absence and presence of ascorbate, ascorbate was added at time *t* to the $O_2^{\cdot-}$-treated sample followed by the addition of excess free [^{3}H]GDP. Apparent rates of $O_2^{\cdot-}$-mediated GDP dissociation (${}^{\text{app } O_2^{\cdot-}}k_{\text{off}}$) and GDP association (${}^{\text{app } O_2^{\cdot-}}k_{\text{on}}$) of Ras were determined by fitting the data to a simple exponential decay and exponential association, respectively.

As xanthine oxidase can continuously produce $O_2^{\cdot-}$ with time, the concentration of $O_2^{\cdot-}$ produced by xanthine oxidase is not fixed during the process of $O_2^{\cdot-}$-mediated GTPase GNE. However, the true first-order rate constant for GDP dissociation of $O_2^{\cdot-}$-mediated Ras and Rap1A (${}^{\text{true } O_2^{\cdot-}}k_{\text{off}}$) can be determined by compensating the experimentally measured ${}^{\text{app } O_2^{\cdot-}}k_{\text{off}}$ according to Equation 2 (52, 53).

$$ {}^{\text{true}O_2^{\cdot-}}k_{\text{off}} = ({}^{\text{app}O_2^{\cdot-}}k_{\text{off}} + {}^{\text{XO pseudo}}k)/[O_2^{\cdot-}] \qquad \text{(Eq. 2)} $$

where ${}^{\text{XO pseudo}}$k represents the pseudo-zero-order rate of xanthine oxidase produced by $O_2^{\cdot-}$.

To determine the dissociation constant for Ras GDP in the presence of $O_2^{\cdot-}$ (${}^{O_2^{\cdot-}}K_D$), fluorescent-based measurements were employed. Xanthine oxidase was incubated for a given time t prior to the addition of Ras. The amount of $O_2^{\cdot-}$ that accumulated over a given time *t* was estimated using Equation 1. The initial first-order rates of $O_2^{\cdot-}$-mediated Ras GDP dissociation (${}^{\text{initial } O_2^{\cdot-}}k_{\text{off}}$) were fit to a single exponential decay to eliminate contributions of $O_2^{\cdot-}$ (continuously produced by the enzymatic action of xanthine oxidase during the assay time period) on $O_2^{\cdot-}$-mediated Ras guanine nucleotide dissociation. This approach avoids the conversion of apparent kinetic values into true values (Equation 2). The estimated ${}^{\text{initial } O_2^{\cdot-}}k_{\text{off}}$ values were then plotted against the concentration of $O_2^{\cdot-}$, and ${}^{O_2^{\cdot-}}K_D$ was determined by fitting the plot to a simple hyperbola.

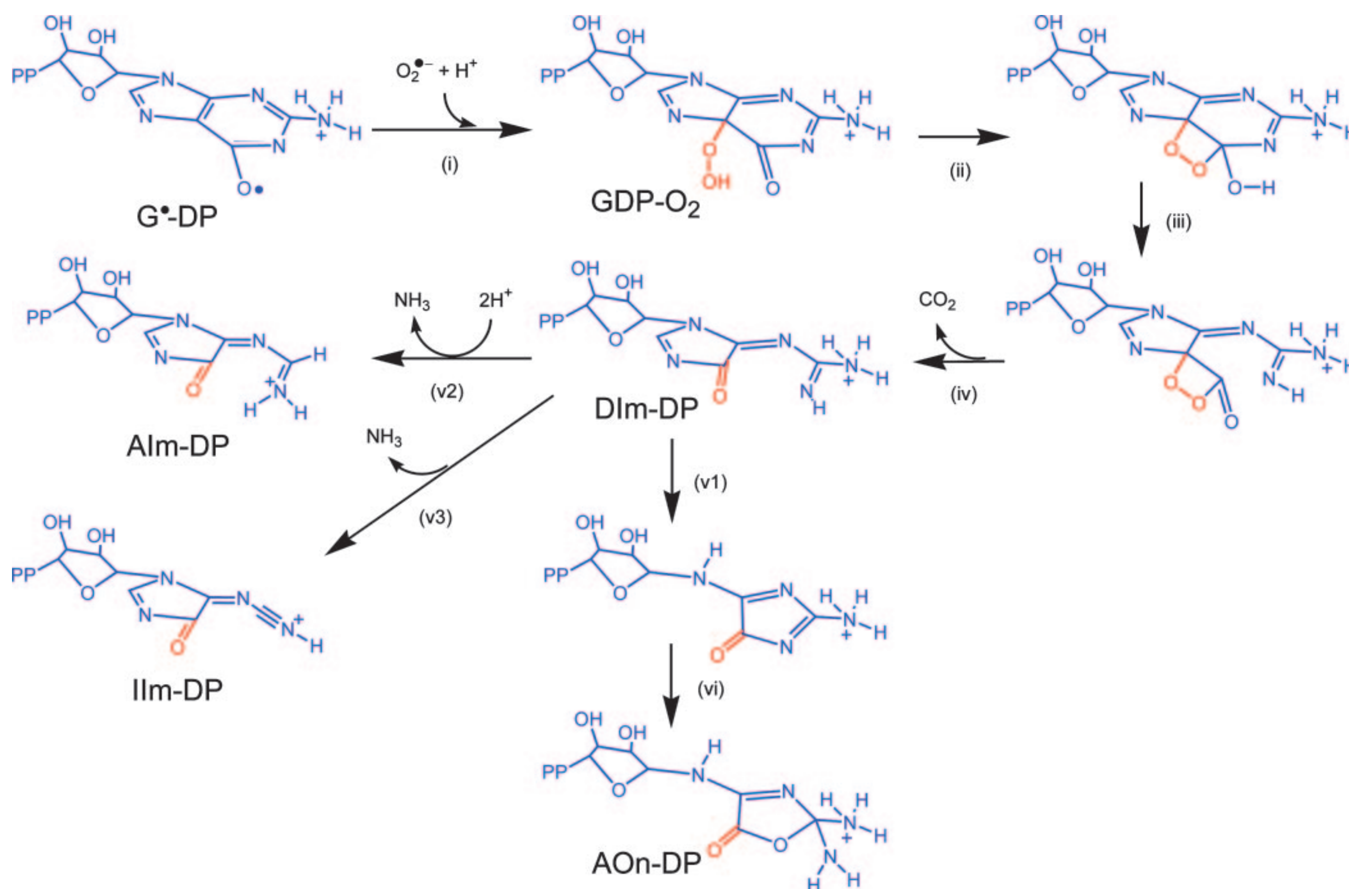

FIG. 4. **Proposed degradation path of $O_2^{\bar{}}$-mediated Ras and Rap1A-released guanine nucleotide products.** GDP and the GDP oxygenated adducts are represented in *blue* and *red*, respectively.

The estimated ${}^{O_2^-}K_D$ value and ${}^{\text{true } O_2^-}k_{\text{off}}$ can be used to determine the value of ${}^{\text{true } O_2^-}k_{\text{on}}$ using Equation 3.

$$ {}^{O_2^-}K_D = {}^{\text{true}O_2^-}k_{\text{off}}/{}^{\text{true}O_2^-}k_{\text{on}} \quad \text{(Eq. 3)}$$

Preparation of Ras and Rap1A-released Nucleotide Nitration Product—An aliquot of Ras or Rap1A protein was introduced into the xanthine oxidase-containing assay solution, and the mixture was incubated for 300 s. The Ras or Rap1A-released nucleotide oxygenation product(s) was separated from Ras or Rap1A protein using a centricon (10-kDa cut off, Millipore).

Mass Spectrometry Analysis—Ras or Rap1A-released nucleotide oxygenation product(s) was acidified with formic acid prior to sample application since MS analysis requires the samples to be positively charged. Thus, only charged ions (*i.e.* $[M+H]^+$) were observed so that the molecular masses determined by MS are 1 Da higher than the molecular masses for the same chemicals at neutral pH (*i.e.* pH 7.5). Briefly, an aliquot of the sample was dissolved in 50% methanol:0.1% formic acid and analyzed within 30 min by nanospray mass spectrometry on an ABI QSTAR-Pulsar QTOF MS spectrometer with a nanoelectrospray source (Applied Biosystems Division, PerkinElmer Life Sciences). The sample (~1 μl) was loaded into a Protana distal coated nanospray needle (Protana, Odense, Denmark), and spectra were acquired over a mass range of 400–500 in MS mode.

RESULTS

In addition to NO (8–14), ROS including O_2^- and H_2O_2 have been shown to modulate Ras activity and Ras-mediated signaling pathways (8, 15–18). Moreover, the Ras-related GTPase, Rap1A, contains a redox-active NKCD motif, is sensitive to NO (19, 20) and has been shown to bind to the O_2^--producing enzyme, NADPH oxidase (40). Given these observations, it is intriguing to speculate that Rap1A activity may be regulated by NADPH oxidase. Although these *in vivo* observations support the possibility that O_2^- mediates regulation of Ras and Ras-related GTPase activities, whether GTPase activity is directly modulated by O_2^-, and if so, the mechanism by which this occurs, has not been elucidated. In this study, we show that O_2^- stimulates guanine nucleotide dissociation of Ras and the Ras-related GTPase Rap1A and provide evidence that the molecular mechanism of O_2^--mediated guanine nucleotide dissociation is similar to that of the NO/O_2-mediated guanine nucleotide dissociation (23).

Kinetics of O_2^- on Ras and Rap1A GNE—Fig. 1 shows that O_2^- generated from xanthine oxidase effectively facilitates guanine nucleotide dissociation from both Ras and Rap1A. Superoxide dismutases catalyze the conversion of O_2^- into O_2 and H_2O_2 and have a major role in defense against oxygen toxicity and regulation of ROS levels in the cell, (54–57). When bovine liver Cu,Zn-superoxide dismutase (~5,000 units) is present, xanthine oxidase-mediated Ras guanine nucleotide dissociation is abolished, indicating that O_2^- generated from xanthine oxidase facilitates Ras guanine nucleotide dissociation.

The estimated true first-order O_2^--mediated mant-GDP dissociation rates for both WT Ras and WT Rap1A (${}^{\text{true } O_2^-}k_{\text{off}} = \sim 3.1 \times 10^{-3}\ \text{s}^{-1}$) are similar to the true NO/O_2 (via $^{\cdot}NO_2$)-mediated mant-GDP dissociation rate (${}^{\text{true NO}}k_{\text{off}}$) of $2.5 \times 10^{-3}\ \text{s}^{-1}$ (22). In addition to the fluorescence-based measurements, a radioactive-based ${}^{\text{true } O_2^-}k_{\text{off}}$ measurement was performed (Fig. 2). The value of ${}^{\text{true } O_2^-}k_{\text{off}}$ ($\sim 3.9 \times 10^{-3}\ \text{s}^{-1}$) for Ras GDP, determined using the radioactive assay, is similar to that determined from the fluorescence-based measurement (Fig. 1). The fluorescence method employing mant-GDP was also applied to determine the value of ${}^{O_2^-}K_D$ (11.3 mM, Fig. 3). The estimated ${}^{O_2^-}K_D$ value for WT Ras was used to determine the true second-order Ras GDP association constant (${}^{\text{true } O_2^-}k_{\text{on}} = 0.27\ \text{M}^{-1}\ \text{s}^{-1}$) following Equation 3. The fast ${}^{\text{true } O_2^-}k_{\text{off}}$ but slow ${}^{\text{true } O_2^-}k_{\text{on}}$ indicates that O_2^- primarily stimulates the dissociation of GDP from WT Ras but not the reassociation of nucleo-

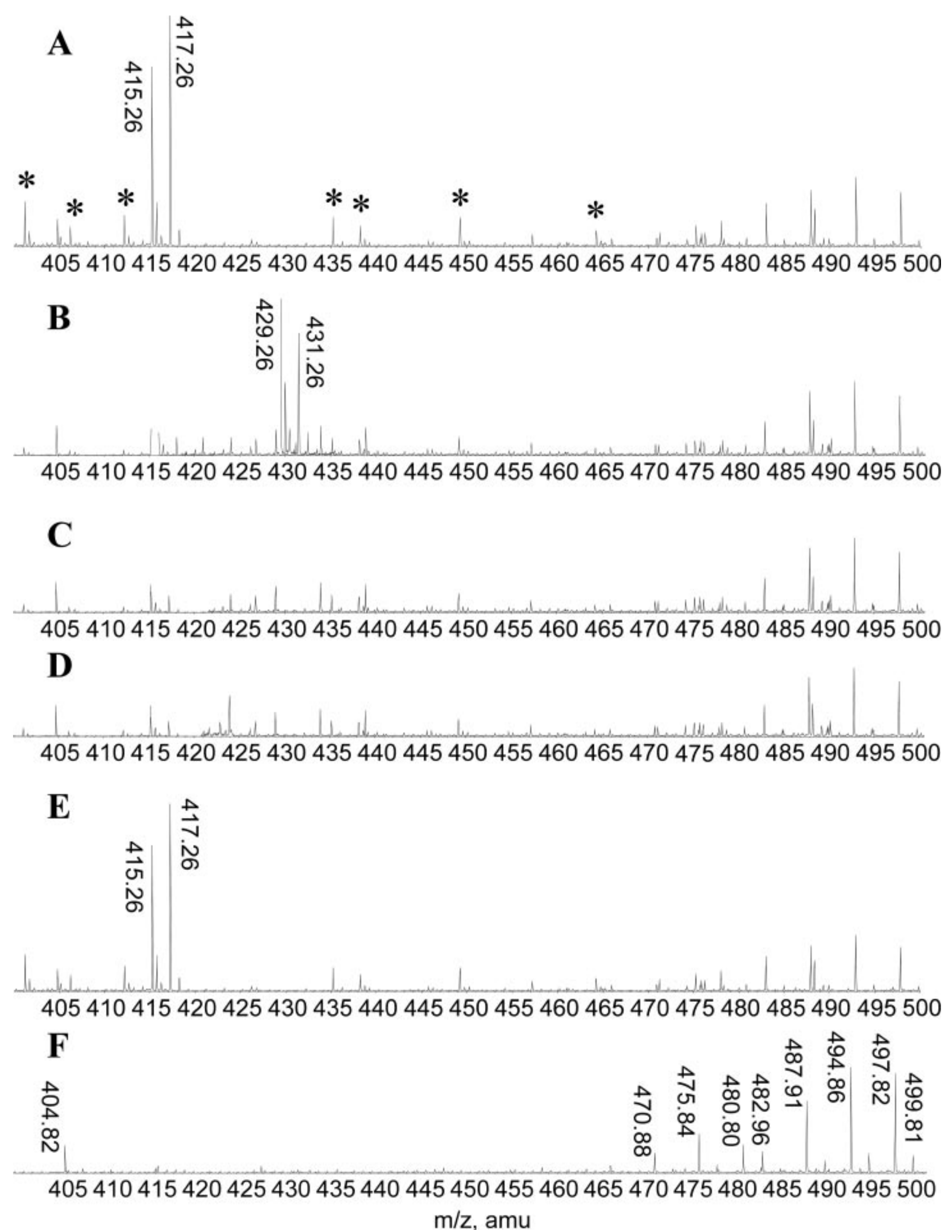

FIG. 5. **Molecular weight determination of $O_2^{\bar{}}$-mediated Ras and Rap1A-bound GDP dissociation products by MS.** Unlabeled GDP was loaded onto WT Ras (*A*), Ras C118S (*C*), Ras F28L (*D*), and WT Rap1A (*E*), whereas radiolabeled [^{13}C-^{15}N]GDP was loaded onto WT Ras (*B*). Sample (*F*) does not contain GTPase and thus serves as a GTPase control. To normalize the MS background, the GTPases samples (WT Ras, Ras variants, and WT Rap1A; 1 μM, 500 μl) and buffer only (500 μl) were transferred to a dialysis bag and dialyzed briefly (5 min) with an identical buffer containing 10 mM $MgCl_2$, 1 mM EDTA, 100 μM GDP, and 5 mM ammonium acetate buffer (pH 7.5). The dialyzed samples (1 μM, 500 μl) were incubated with xanthine oxidase (unit activity: 0.5 μM^{-1} s^{-1}) in the presence of xanthine (1 mM) for 300 s to complete the reaction. Fractions containing the nucleotide products from the samples *A*–*E* as well as the control *F* were prepared and analyzed by MS as described under "Experimental Procedures." For each assigned chemical adduct, two additional satellite peaks can be observed due to the presence of isomers of the assigned chemical adducts (23). Unassigned minor peaks are noted as *.

tides (both GDP and GTP) to WT Ras, suggesting that $O_2^{\bar{}}$ produces nucleotide-deficient Ras (apo-Ras$^{O_2^{\bar{}}}$).

To directly determine the rate of Ras guanine nucleotide association in the presence of $O_2^{\bar{}}$, we performed a radioactive-based assay. An excess of free [^{3}H]GDP (100 mM) was reintroduced to $O_2^{\bar{}}$-treated [^{3}H]GDP-loaded Ras after an extended time passed (500 s in Fig. 2). A slow increase in radioactivity, due to the rebinding of [^{3}H]GDP to Ras, was observed (Fig. 2), suggesting that, after a prolonged time period (*i.e.* 500 s), ${}^{\text{true } O_2^{\bar{}}}k_{on}$ slowly increases. A possible explanation for these results is that the fast ${}^{\text{true } O_2^{\bar{}}}k_{off}$ and slow ${}^{\text{true } O_2^{\bar{}}}k_{on}$ gradually decay over time, and thus, the intrinsic rates of Ras guanine GDP dissociation (${}^{int}k_{off}$) and GDP association (${}^{int}k_{on}$) slowly recover (51). However, the rate of Ras GDP association is greatly enhanced by the addition of a radical scavenger, 1 mM ascorbate (Fig. 2) or 5 mM GSH (results not shown), respectively, consistent with previous results obtained for NO/O_2-mediated Ras GNE (22). Thus, it is likely that ascorbate and GSH quench $O_2^{\bar{}}$ (and possibly an $O_2^{\bar{}}$-relevant Ras-radical species) in the assay mixture that maintains the fast ${}^{\text{true } O_2^{\bar{}}}k_{off}$ but slow ${}^{\text{true } O_2^{\bar{}}}k_{on}$ to produce apo-Ras$^{O_2^{\bar{}}}$, which results in the recovery of ${}^{int}k_{off}$ and ${}^{int}k_{on}$, thereby facilitating Ras GNE.

Given that xanthine oxidase produces H_2O_2 as well as $O_2^{\bar{}}$ in the presence of O_2 and xanthine (49), we examined whether H_2O_2 can promote Ras guanine nucleotide dissociation. As shown in Fig. 1, we did not observe an enhancement in the rate of guanine nucleotide dissociation from Ras in the presence of 1% H_2O_2, indicating that H_2O_2 does not facilitate Ras guanine nucleotide dissociation. To further examine the effect of H_2O_2 produced by xanthine oxidase on Ras guanine nucleotide dissociation, we added bovine liver catalase. Catalase converts H_2O_2 to H_2O and O_2 (58). If H_2O_2 facilitates Ras guanine nucleotide dissociation, the addition of catalase to the xanthine oxidase assay should inhibit the rate of Ras guanine nucleotide dissociation. The addition of an excess amount of catalase (~1,000 units) did not impede but rather slightly facilitated the rate of the xanthine oxidase-mediated Ras guanine nucleotide dissociation (Fig. 1). It is difficult to rationalize the minor stimulation of the catalase-mediated xanthine oxidase-coupled Ras guanine nucleotide dissociation rate as multiple factors may be involved. For example, removal of H_2O_2 using catalase may enhance the release of H_2O_2 (xanthine oxidase end product) from xanthine oxidase, which in turn may facilitate the turnover rate of

xanthine oxidase to produce $O_2^{\cdot-}$ in addition to H_2O_2. Generation of more $O_2^{\cdot-}$ may then further facilitate $O_2^{\cdot-}$-mediated Ras guanine nucleotide dissociation. Notwithstanding, since catalase did not impede xanthine oxidase-mediated Ras guanine nucleotide dissociation, xanthine oxidase-coupled Ras guanine nucleotide dissociation can be attributed to the presence of $O_2^{\cdot-}$, not H_2O_2. Our findings that the direct addition of H_2O_2 (1%) does not facilitate Ras guanine nucleotide dissociation is in agreement with these results (*vide supra*).

It has previously been shown that a protein thiol (*i.e.* bovine serum albumin Cys^{34}; BSA-S^{34}) can be oxidized by H_2O_2 to produce a protein sulfenic acid adduct (*i.e.* BSA-S^{34}-OH) (59). If the Ras Cys^{118} thiol oxidizes to sulfenic acid (Ras-S^{118}-OH) due to the presence of H_2O_2, the formation of a Ras sulfenic acid adduct does not facilitate GDP dissociation from Ras (Fig. 1). Consistent with these observations, we have previously shown that modification of the Ras Cys^{118} thiol by NO to produce *S*-nitrosylated Ras (Ras-SNO) does not promote NO/O_2-mediated Ras guanine nucleotide dissociation (22, 23).

Characterization of End Products of $O_2^{\cdot-}$-mediated Ras Guanine Nucleotide Dissociation—Intriguingly, recent studies showed that the reaction of thiols (GSH or cysteine) with $O_2^{\cdot-}$ produces a thiyl radical ($GS^{\cdot}$) (60–62). Given these results, it is possible that $O_2^{\cdot-}$ can react with the Ras redox-active Cys^{118} thiol to produce Ras-$S^{118\cdot}$. If this is the case, the mechanism of $O_2^{\cdot-}$-mediated Ras guanine nucleotide dissociation is expected to be similar to that of NO/O_2 (via $^{\cdot}NO_2$)-mediated Ras guanine nucleotide dissociation (23) since both $O_2^{\cdot-}$, and $^{\cdot}NO_2$ can commonly react with Ras Cys^{118} thiol to produce Ras-$S^{118\cdot}$. A detailed mechanism for Ras-$S^{118\cdot}$-initiated Ras guanine nucleotide dissociation has been proposed (23). In this mechanism, electron transfer from the Ras-bound GDP guanine base to Ras-$S^{118\cdot}$ via Phe^{28} side chain produces a Ras-bound $G^{\cdot+}$-DP, which is subsequently converted into Ras-bound $G^{\cdot}$-DP. This radical reaction process is likely to perturb the aromatic-aromatic interaction between the Phe^{28} side chain and the Ras-bound guanine base as well as interactions between Ras and the guanine base of bound GDP, resulting in the release of GDP from Ras. Consistent with this premise, the use of Ras variants containing mutations in the redox-active Ras residues Cys^{118} and Phe^{28} (23) abolish $O_2^{\cdot-}$-mediated guanine nucleotide dissociation from Ras (Fig. 1), suggesting that Ras residues Cys^{118} and Phe^{28} play a key role in $O_2^{\cdot-}$-mediated Ras guanine nucleotide dissociation, and the process of $O_2^{\cdot-}$-mediated Ras guanine nucleotide dissociation and GNE (if ascorbate is present) may be radical-based, similar to the process of NO/O_2-mediated Ras guanine nucleotide dissociation (23).

The original study (23) that led to our proposed radical-based mechanism was conducted in the presence of NO/O_2 to generate $^{\cdot}NO_2$, where we observed that Ras-bound $G^{\cdot}$-DP can react with $^{\cdot}NO_2$ to produce 5-nitro-GDP, which is subsequently decarboxylated to produce NIm-DP as an end product. The end products of $O_2^{\cdot-}$-mediated guanine nucleotide dissociation of Ras and Ras-related GTPases, however, are likely to differ from those produced by $^{\cdot}NO_2$-mediated guanine nucleotide dissociation of Ras and Ras-related GTPases (5-nitro-GDP) since reaction of the proposed $G^{\cdot}$-DP with $O_2^{\cdot-}$ will generate nucleotide oxygenation products. However, as suggested in our previous study (23), since the C_5 site of Ras-bound $G^{\cdot}$-DP is exposed to solvent (hence $O_2^{\cdot-}$ is accessible to the C_5 site of Ras-bound $G^{\cdot}$-DP), whereas the C_8 site of Ras-bound $G^{\cdot}$-DP is not solvent-accessible (63–68), 5-oxo-GDP is likely to be the dominant product formed over 8-oxo-GDP (Fig. 4). The proposed reaction process is shown in Fig. 4, where reaction of the $G^{\cdot}$-DP C_5 with $O_2^{\cdot-}$ produces 5-oxo-GDP (*step i*). 5-Diamino-4*O*-imidazolone ribose diphosphate (DIm-DP) is formed after decarboxylation of 5-oxo-GDP (*step iv*), which can be further degraded into 5-amino-oxazolone ribose diphosphate (AOn-DP) (69) (*steps v1* and *vi*), 5-amino-4*O*-imidazolone ribose diphosphate (AIm-DP) (*step v2*), and/or 5-imino-4*O*-imidazolone ribose diphosphate (IIm-DP) (*step v3*).

Adducts released from Ras upon exposure to xanthine oxidase were analyzed by MS. Two main peaks with molecular masses of 415.26 and 417.26 Da were observed that correspond to end products of $O_2^{\cdot-}$-treated Ras (Fig. 5*A*). Given that the predicted molecular masses of AIm-DP and IIm-DP are 415.26 and 417.26 Da, respectively, we putatively assigned the two Ras-released GDP-O_2 derivatives as AIm-DP and IIm-DP. As anticipated, we were unable to detect the MS peak corresponding to AOn-DP since our MS sample analysis was performed within 30 min after the sample was prepared, and the formation of AOn-DP from DIm-DP requires a prolonged incubation time (>24 h) in the dark (69). We were also unable to detect MS peaks corresponding to the molecular weight of 8-oxo-GDP as well as possible degradation products of 8-oxo-GDP. The inability to detect these adducts is consistent with our premise that the C_8 site of $G^{\cdot+}$-DP is not exposed to solvent so that the reaction product of the $G^{\cdot}$-DP C_8 with $O_2^{\cdot-}$, 8-oxo-GDP, is unlikely to be produced and detected.

To further assess whether 5-oxo-GDP is derived from the Ras-bound GDP upon treatment of Ras with $O_2^{\cdot-}$ (employing xanthine oxidase), MS analyses were performed on [^{13}C-^{15}N]GDP-loaded Ras after treatment with $O_2^{\cdot-}$. When uniformly labeled [^{13}C-^{15}N]GDP-loaded Ras was treated with $O_2^{\cdot-}$, the detected molecular masses of the $O_2^{\cdot-}$-treated Ras-released oxygenated-nucleotide derivatives were found to be 429.26 and 431.26 Da (Fig. 5*B*). The molecular mass difference between unlabeled GDP and [^{13}C-^{15}N]GDP is 15, whereas the molecular mass difference between the oxygenated-nucleotide derivatives released from Ras loaded with unlabeled GDP and Ras loaded with [^{13}C-^{15}N]GDP upon treatment of Ras with $O_2^{\cdot-}$ is 14. These results indicate that the oxygenated-nucleotide derivatives released from Ras upon treatment of $O_2^{\cdot-}$ originate from Ras-bound GDP as the $^{13}C_6$ atom associated with the guanine base-O_2 is decarboxylated to produce DIm-DP (Fig. 4, *step iv*), which would give rise to a loss of the ^{13}C atom from Ras-bound [^{13}C-^{15}N]GDP upon treatment with $O_2^{\cdot-}$.

We have previously provided evidence that the Ras residues Cys^{118} and Phe^{28} play an important role in NO/O_2-mediated GNE on Ras (23), and mutation of these residues (Cys^{118} and Phe^{28}) does not enhance GNE in the presence of NO/O_2 (23). Therefore, if the fundamental mechanism of $O_2^{\cdot-}$-mediated Ras guanine nucleotide dissociation is similar to that of $^{\cdot}NO_2$-mediated Ras guanine nucleotide dissociation, we would anticipate that the Ras-released 5-oxo-GDP derivatives, AIm-DP and IIm-DP, will not be produced upon treatment of Ras variants C118S and F28L with $O_2^{\cdot-}$. Consistent with this premise, the MS peaks assigned putatively as AIm-DP and IIm-DP were not observed in samples prepared from both C118S and F28L (Fig. 5, *C* and *D*), indicating that similar to NO/O_2-mediated Ras guanine nucleotide dissociation (23), Ras residues Cys^{118} and Phe^{28} play key roles in $O_2^{\cdot-}$-mediated dissociation of Ras-bound GDP, in agreement with the kinetic results shown Figs. 1 and 2.

In addition to Ras, end products of $O_2^{\cdot-}$-mediated Rap1A guanine nucleotide dissociation were also examined. The MS peaks 415.26 and 417.26 Da that are identical to those of Ras have been observed (Fig. 5*E*), suggesting that the mechanism of $O_2^{\cdot-}$-mediated guanine nucleotide dissociation of the redox-active NKCD motif-containing GTPase, Rap1A, is likely similar to that of Ras.

DISCUSSION

Numerous studies have now implicated the free radicals NO and $O_2^{\cdot-}$ in both Ras and Ras superfamily GTPase signaling pathways (8–20). To better understand the role of redox agents in Ras-mediated signaling, we have conducted a series of kinetic and spectroscopic studies and recently proposed a mechanism of NO-mediated Ras guanine nucleotide dissociation in the presence of O_2. According to this mechanism, $^{\cdot}NO_2$ (the reaction product of NO with O_2) promotes Ras guanine nucleotide dissociation through the radical-based conversion of Ras-bound GDP into 5-nitro-GDP, which is further degraded into NIm-DP (23).

In this study, we show that $O_2^{\cdot-}$ facilitates guanine nucleotide dissociation of Ras and the Ras-related GTPase Rap1A. Based on results obtained from this work, the basic mechanism of $O_2^{\cdot-}$-mediated guanine nucleotide dissociation from the NKCD motif-containing GTPases, Ras and Rap1A, appears similar to that of NO/O_2-mediated Ras guanine nucleotide dissociation (23). However, the reaction end product of $O_2^{\cdot-}$-mediated Ras guanine nucleotide dissociation differs from that of the NO/O_2-mediated Ras guanine nucleotide dissociation. Although $O_2^{\cdot-}$-mediated Ras guanine nucleotide dissociation produces 5-oxo-GDP, NO/O_2-mediated Ras guanine nucleotide dissociation produces 5-nitro-GDP (23).

On the basis of these results in conjunction with our previous study (23), we propose the following mechanism for $O_2^{\cdot-}$-mediated Ras guanine nucleotide dissociation. (*a*) $O_2^{\cdot-}$ reacts with the GTPase Cys^{118}-SH to produce a GTPase-$S^{118\cdot}$ radical intermediate. Cys^{118}-SH may be deprotonated prior to reaction with $O_2^{\cdot-}$ to produce GTPase-$S^{118\cdot}$. (*b*) The GTPase-$S^{118\cdot}$ then withdraws an electron from the guanine nucleotide base to produce $G^{\cdot+}$-DP. Similar to the mechanism described for $^{\cdot}NO_2$-mediated Ras guanine nucleotide dissociation (23), the Phe^{28} side chain may serve as an electron conduit for this process. $G^{\cdot+}$-DP is thus expected to be formed and converted to $G^{\cdot}$-DP by elimination of H^+ from the N_1 atom of $G^{\cdot+}$-DP. The process disrupts key hydrogen bond interactions as well as the n-π interaction between the GTPase and its ligand nucleotide. (*c*) The Ras-bound $G^{\cdot}$-DP can then react with $O_2^{\cdot-}$ to produce 5-oxo-GDP, releasing 5-oxo-GDP from the GTPase. Depending on the experimental conditions, the Ras-released 5-oxo-GDP is further degraded into oxygenated-nucleotide derivatives, such as AIm-DP or/and IIm-DP.

We also show that $O_2^{\cdot-}$-mediated Ras signaling is unidirectional (*i.e.* guanine nucleotide dissociation but not association is enhanced) in the absence of a radical scavenging agent. $O_2^{\cdot-}$ effectively dissociates GDP from Ras in the form of 5-oxo-GDP but does not enhance association of GDP. Like NO/O_2-mediated Ras guanine nucleotide dissociation (23), treatment of Ras-GDP with $O_2^{\cdot-}$ promotes GDP dissociation to produce a nucleotide-deficient radical form of Ras (apo-$Ras^{O_2^{\cdot-}}$) that cannot efficiently associate with guanine nucleotide ligands. Moreover, results from our kinetic studies indicate that apo-$Ras^{O_2^{\cdot-}}$ can be converted to an active guanine nucleotide binding form of Ras by the addition of a radical scavenger (*i.e.* ascorbate or GSH) in the presence of GDP. On the basis of our results and analyses, we propose that both $O_2^{\cdot-}$ and a radical quenching agent are required to complete Ras GNE (both Ras guanine nucleotide dissociation and association). Therefore, given the cellular abundance of GTP over GDP, the combined action of $O_2^{\cdot-}$ and a radical scavenger could lead to Ras activation *in situ*. In cells, GSH is present at high levels and may function as a radical scavenger. Natural quenching agents ascorbate (vitamin C) and tocopherol (vitamin E) may also serve as additional radical scavenging agents in cells (34) since both chemicals are able to quench most cellular radical species. The results further suggest that $O_2^{\cdot-}$-mediated Ras activation requires synergy with a radical scavenger, such as GSH or ascorbate.

The mechanism proposed for $O_2^{\cdot-}$-mediated Ras guanine nucleotide dissociation is likely to be applicable to other redox-active NKCD motif-containing GTPases since guanine nucleotide dissociation of the NKCD motif-containing Rap1A is also facilitated by $O_2^{\cdot-}$ and its end products are identical to those of Ras. However, whether the GTPases are activated by these redox agents *in vivo* will be highly dependent on their ability to colocalize with sources of NO or $O_2^{\cdot-}$. Since Rap1A colocalizes with NAPH oxidase (40), we would predict that Rap1A will be activated by $O_2^{\cdot-}$ under conditions in which NADPH oxidase is stimulated to produce $O_2^{\cdot-}$ in cellular locations containing Rap1A.

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