Activation of c-Raf Kinase by Ultraviolet Light

REGULATION BY RETINOIDS*

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The present study highlights retinoids as modulators of c-Raf kinase activation by UV light. Whereas a number of retinoids, including retinol, 14-hydroxyretinol, anhydroretinol (AR), and retinoic acid bound the c-Raf cysteine-rich domain (CRD) with equal affinity in vitro as well as in vivo, they displayed different, even opposing, effects on UV-mediated kinase activation; retinol and 14-hydroxyretinol augmented responses, whereas retinoic acid and AR were inhibitory. Oxidation of thiol groups of cysteines by reactive oxygen, generated during UV irradiation, was the primary event in c-Raf activation, causing the release of zinc ions and, by inference, a change in CRD structure. Retinoids modulated these oxidation events directly: retinol enhanced, whereas AR suppressed, zinc release, precisely mirroring the retinoid effects on c-Raf kinase activation. Oxidation of c-Raf was not sufficient for kinase activation, productive interaction with Ras being mandatory. Further, canonical tyrosine phosphorylation and the action of phosphatase were essential for optimal c-Raf kinase competence. Thus, retinoids bound c-Raf with high affinity, priming the molecule for UV-reactive oxygen species-mediated changes of the CRD that set off GTP-Ras interaction and, in context with an appropriate phosphorylation pattern, lead to full phosphotransferase capacity.

The c-Raf proto-oncogene is essential for cell growth, differentiation, and survival. Its major downstream effector is the mitogen-activated protein kinase (MAPK)1 (1, 2) that elicits a complex set of cytosolic (3–5) as well as nuclear signals (6–9). The molecular mechanism of c-Raf activation has not yet been fully elucidated (for reviews, see Refs. 10 and 11). That growth factors and cytokines, as well as UV and ionizing radiation, all lead to the activation of the c-Raf/MAPK pathway has been amply demonstrated (12–15). Receptor protein tyrosine kinase, ligated by their respective growth factors, dimerize, become autophosphorylated, and recruit adapter molecules (Grb2) and the nucleotide exchange factor SOS to the cell membrane. The further assembly of GTP-bound Ras enables c-Raf to translocate from the cytosol to the plasma membrane, where an as yet unidentified mechanism bestows competence on c-Raf to activate the MAPK cascade (16). Phosphorylation of tyrosine residues (e.g. Tyr-340 and Tyr-341 (17)) and dephosphorylation of serine residues (Ser-259 and Ser-621 (18)) are believed to lock c-Raf into the optimally competent form.

For docking with Ras, two important contact sites in the regulatory domain of c-Raf have been identified, one centered on the stretch of amino acids 51–131, the other contained within the CRD (19, 20). What remains to be identified is the initial molecular event that triggers cytosol-to-membrane translocation. Whether this involves changes in the phosphorylation pattern and consequent changes in the conformation of the regulatory domain is still unclear. The participation of lipid mediators in the activation of c-Raf has been suspected because of structural similarities with the PKC family of serine/threonine kinases (21, 22), which harbor lipid binding sites in their CRD tandem repeats (23–25). Bound phosphatidylinerse enhanced PKC activity (26). Several groups have identified lipid binding sites in the regulatory and catalytic regions of c-Raf (27–30). Interestingly, Romero and colleagues (30) suggested that phosphatidic acid mediated c-Raf translocation from cytoplasm to membrane independently of its association with Ras.

Besides the classical RTPK signal chain, alternative activation signals exist. Ultraviolet light and ionizing irradiation as well as oxidizing agents lead to c-Raf/MAPK activation (13–15). To understand the biological significance, it is worth remembering that macrophages naturally produce substantial concentrations of hydrogen peroxide and that reactive oxygen species (ROS) are produced in every cell type by mitochondria as well as by dedicated enzyme systems. The changing view is that ROS, like nitric oxide, serve as normal intracellular messengers (31–34). Also long known, the potent activating capacity of ultraviolet irradiation rests on the intracellular production of ROS (35, 36).

Whereas the chemistry of oxidative activation of serine/threonine kinases is poorly understood, it stands to reason that ROS target the most susceptible groups in c-Raf, namely the thiols of cysteines, assuming a direct chemical modification and not activation of an upstream factor. Direct attack by ROS is all the more likely, since six cysteine residues are clustered within a stretch of 50 amino acids of the regulatory domain, all susceptible to oxidation. The questions of what changes the CRD may undergo during UV activation and how retinoids regulate such chemical changes are addressed in the present report. If the biology of vitamin A were a guide (37–40), the expectation would be that hydroxylated retinoids (retinol and 14HRR) enhanced, whereas anhydroretinol attenuated, the UV effects. This prediction was borne out.
As previously shown, vitamin A functions as regulatory co-factor for redox regulation of c-Raf and other serine/threonine kinases (41, 42). A single site capable of binding several natural vitamin A metabolites at nanomolar affinity was mapped to the CRD. Since bound retinoids influenced redox activation (42), the importance of this domain as a primary target for oxidation was suggested. Further, two zinc coordination centers exist per CRD, each composed of three thiol groups of cysteines and one imino group of histidine. Chemistry predicts that oxidation of one or more thiols would compromise the integrity of the zinc finger. Precedents that zinc finger structures serve as redox sensors of enzymes exist in bacteria, and the related structures in PKC isoforms (42, 43) have been suggested. In the present report, the CRD of c-Raf is shown to be an important primary target for oxidation during UV transduction of c-Raf activity by ROS generated during UV irradiation. Several findings in this report will highlight our conclusion: the redox-sensitive hinge of the CRD is required for activation of c-Raf kinase by UV light.

MATERIALS AND METHODS

Retinoids—All-trans isomers of AR and 14HRR were synthesized as described previously (37, 44). Retinol was purchased from Sigma and purified by high pressure liquid chromatography. [3H]Retinol was purchased from PerkinElmer Life Sciences.

Immunological Reagents and Chemicals—Anti-FLAG® M2-agarose affinity gel, herbinycin A, okadaic acid, vanadate, and pericil acid were obtained from Sigma. Rabbit antibody to the c-Raf C-terminal peptide (136LTTHNFA-VDWSNIRQLL195; this peptide contains the natural Trp-186 residue, responsible for fluorescence emission) were constructed as previously described (37, 44).

Bacteria Growth and Protein Purification—E. coli BL21/DE3 strain of the c-Raf CRD (35, 36) was prepared as GST fusion protein in the BL21/DE3 strain of E. coli (45). Briefly, 5 × 10^6 cells were plated in 60-mm dishes the day before transfection. 2 h prior to transfection, the medium was replaced with fresh growth medium (Dulbecco’s modified Eagle’s medium (high glucose) supplemented with 10% fetal calf serum). 12 μg of DNA/dish were mixed with CaCl₂ and phosphate buffer to form a DNA precipitate, which was then resuspended over the cells. The day after transfection, the medium was removed and the cells were washed twice with 3 ml each of phosphate-buffered saline (PBS). The cells were cultured with 2 ml of retinoid-free, phenol red-free Dulbecco’s modified Eagle’s medium (high glucose) for 2½ days prior to activation. UV irradiation was performed for 2 min at 400 milliwatts/cm² using the 312-nm wavelength. Cultures were incubated at 37 °C for 10 min after irradiation and harvested.

c-Raf Immunoprecipitation/Kinase Assay—Cells were lysed in 100 μl lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1% Triton X-100, 25 μg/ml each leupeptin and aprotinin, 30 mM β-glycerophosphate, 30 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 1 mM vanadate). The lysates were preclarified with 30 μl of a 50% (v/v) protein G-agarose slurry, and the c-Raf-c-FLAG M2 antibody was precipitated using 30 μl of anti-FLAG M2 affinity gel (Sigma).

AMS Trapping of Free Thiols in Vivo—Cells transfected with Flag-c-Raf CRD were UV-irradiated, treated with 1 or 10 mM AMS, or left untreated and incubated at 37 °C for 10 min. The medium was removed, cells were washed with PBS, and 150 μl of 100 mM iodoacetamide in permeabilization buffer (lysing buffer containing 20 μg/ml digitonin instead of 1% Triton X-100) was added. After incubation for 10 min at 37 °C, the reaction was terminated by the addition of 1% Triton X-100, and cells were frozen in liquid nitrogen. The Flag-c-Raf CRD protein was immunoprecipitated using anti-FLAG M2 antibody gel and analyzed as described above.

Bacteria Growth and Protein Purification—The Flag-c-Raf was a gift from Dr. Roger J. Davis (University of Washington, Seattle, WA), glutathione-S-transferase (GST) and Flag-human c-Raf CRD (35, 36) were purchased from PerkinElmer Life Sciences. 

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In Vivo Retinol Binding Assays—COS-7 cells were transfected with FLAG-c-Raf cDNA and cultured in retinol-free medium as indicated above. Cells were preincubated with 100 nM retinol, 14HRR, AR, RA, or phosphatidylserine for 15 min prior to the addition of 5 nM [3H]retinol for 30 min and harvested after washing with PBS containing 0.5% bovine albumin. Extracts were prepared by repeated freeze thawing in 0.25 M Tris, pH 8, supplemented with 25 μg/ml each leupeptin and aprotinin, 30 mM β-glycerophosphate, 30 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 1 mM vanadate. c-Raf immunoprecipitates were washed extensively with PBS containing 0.5 M NaCl and 0.5% bovine albumin, and incorporated counts were measured by liquid scintillation counting. Results were expressed as differentials between immunoprecipitates of transfected and nontransfected cultures.

Zinc Release Assay—A solution of 250 nM of GST-c-Raf CRD fusion protein in PBS was UV-irradiated at 312 nm for 2 min in the absence or presence of an equimolar concentration of retinol, AR, or retinol plus AR, followed by the addition of 3 μM TSQ. The changes in the fluorescence emission spectrum of TSQ (excitation λmax = 335 nm) were monitored from 290–600 nm.

RESULTS

Retinoids Bind the c-Raf CRD in Vitro—Two spectrofluorimetric methods, state of the art in the field (41, 49), were employed to measure binding of retinoids to the c-Raf CRD: quenching and enhancement. Quenching is based on the decrease in the intrinsic protein fluorescence due to resonance energy transfer to a suitable bound ligand. Fluorescence emanated from the natural Trp-186 residue. Preliminary evidence3 suggested that retinol bound nearby at the second zinc chelation center formed by Cys residues 165, 168, and 184, in cooperation with His-139. Enhancement, or increase in the intrinsic fluorescence of the ligand/retinoids, is predicated on the movement of the ligated retinoids from the aqueous phase to the hydrophobic environment of the receptor/CRD protein.

We have previously shown that retinol bound c-Raf and certain PKC CRDs with nanomolar affinity (41). To test for binding of other natural retinol metabolites, solutions of bacterially expressed GST-c-Raf-CRD fusion protein were excited at 280 nm, and the changes in protein fluorescence emission spectra were recorded, brought about by the additions of stoichiometric amounts of retinoids. As illustrated in Fig. 1A, the fluorescence intensity of c-Raf CRD decreased in the presence of 14HRR, AR, or RA, indicating binding. The signal was particularly prominent for 14HRR, followed by AR, and moderately decreased by RA. In distinction from retinol, no detectable fluorescence resonance energy transfer signal was generated by the last three retinoids, probably due to their poor fluorescence properties.

Binding of the retinoids, retinol, 14HRR, and AR, to the c-Raf CRD was confirmed by the fluorescence enhancement method. Fig. 1B shows the idiosyncratic increases in the emission spectrum intensities that each retinoid produced when ligated to an equimolar amount of c-Raf CRD. The magnitude of the enhancement differed for each retinoid, reflecting the differences in their chromophore properties. RA, a poorly fluorescent retinoid, could not be evaluated by this method.

To determine the binding constants, titrations of retinol, 14HRR, AR, and RA were performed (Fig. 2). c-Raf CRD (250 nM) was excited at 280 nm, and its fluorescence emission was monitored at 330 nm after the addition of each 25 nM increment of retinoid from a 75 μM stock solution in methanol. The binding curves, corrected for inner filtering, indicated that binding was saturable. When applied to a nonlinear curve fitting theorem developed by Norris et al. (49), the apparent dissociation constants in the nanomolar range were computed (Table I). Titration using GST as specificity control yielded a flat line (data not shown), no binding having taken place. Together, the results demonstrate high affinity binding of the retinoids to the CRD in vitro.

Retinoids Bind c-Raf in Vivo—Retinol has been shown to bind c-Raf in vivo (41). To demonstrate binding of other reti-
tested, extending our previous findings (41, 42). A receptor site harbors a receptor site with equal specificity for four retinoids (as published previously (41)). When compared with c-Raf kinase capacity in vivo demonstrated binding of retinoids to the CRD of c-Raf and act as functional antagonists.

Because these effects were at least partially attributable to c-Raf kinase regulation in vivo (41), we tested whether retinol and AR would behave as functional antagonists in c-Raf kinase assays. As shown in Fig. 5, the inhibitory effect of AR on retinol, when used at equimolar concentrations, was evident. These results indicate that the retinoids compete for binding to c-Raf and act as functional antagonists.

**UV Activation of c-Raf Is Not Reversible by Reduction**—UV irradiation generates ROS (51–53). At low levels, these radicals are not only tolerated by cells but are important for normal signal transduction (33, 34). Since the hallmark of physiological activating signals is their reversibility, we investigated whether UV activation of c-Raf was abrogated by reducing agents. Fig. 6 illustrates 67% inhibition of c-Raf activation by the addition of L-N-acetylcycteine (NAC) at the time of UV stimulation followed by incubation for 10 min (compare lane 3 with lane 5). However, once fully activated for 10 or 15 min, c-Raf could no longer be influenced by the addition of 1 mM NAC for 1 min (compare lane 3 with lane 6, and compare lane 4 with lane 7), indicating that a complex mechanism controlled the down-regulation of the kinase activity, requiring more than a simple increase in the intracellular reducing power of the cell.

**UV Irradiation Directly Affects c-Raf in Vivo via Oxidation by ROS**—To test whether ROS generated during UV stimulation had a direct effect on c-Raf in vivo, we used the thiol-binding probe AMS to test for the modification of thiol groups (47, 54).

| Retinoid | c-Raf CRD $K_d$ (nM) |
|----------|----------------------|
| Retinol  | 22.0 ± 3.5           |
| 14HRR    | 30.7 ± 4.3           |
| AR       | 36.4 ± 11            |
| RA       | 19.8 ± 4             |
This assay is predicated on the alkylation of free thiol groups, but not cysteine residues, by iodoacetamide. Reduction of disulfide, generated during UV/ROS exposure, restores free thiol groups that are now available for reaction with AMS. Each bound AMS residue theoretically increases the molecular mass of the protein by 490 daltons. The altered size of FLAG-c-Raf CRD protein was assessed by electrophoresis in SDS-PAGE under nonreducing conditions, although conformational changes could also contribute. The product of UV-irradiated cells migrated significantly more slowly, consistent with AMS modifications of the thiol groups, compared with the reference protein from untreated cells (Fig. 7A).

**UV Irradiation of c-Raf CRD Results in Zinc Release**—The c-Raf CRD comprises two zinc-coordinated centers, one of which is formed by the three thiol groups of Cys-165, Cys-168, and Cys-184 and one imino group of His-139, whereas the other is formed by Cys-152, Cys-155, Cys-176, and His-172. Because thiol groups were modified following UV irradiation, we predicted that Zn$^{2+}$ would be released. Furthermore, we investigated to what extent retinoids might modulate the UV-mediated oxidation and zinc release from the c-Raf CRD. Fig. 7B illustrates that the c-Raf-CRD fusion protein indeed shed its zinc upon UV irradiation, as determined by the binding of liberated zinc to TSQ, a zinc-sensitive fluorescent probe, in agreement with recent in vivo observations.3 In the presence of retinol, the fluorescence emission of TSQ was significantly enhanced, indicating that at similar ROS output, additional zinc nevertheless was released. By contrast, AR inhibited UV-mediated release of zinc ions from the c-Raf CRD. These results are consistent with the postulated function of retinoids as redox regulators. They strengthen the idea that the CRD represents the primary target of UV irradiation and oxidation.2 The hierarchy of in vitro effects of retinoids paralleled those seen in vivo precisely. These results for the first time also imply a central role of zinc ions in the regulation of c-Raf kinase.

**The c-Raf CRD Is Essential for c-Raf Function**—c-Raf shares the structural CRD motif with members of the PKC family (21).
Furthermore, the homologous domains in the PKC family bearing the diacylglycerol and phorbol ester binding sites have been implicated in the redox regulation of kinase function but do not promote interaction with Ras. To investigate whether these domains are functionally interchangeable, we replaced the c-Raf CRD domain with the PKC/1C1B domain. COS-7 cells, transfected with wild type FLAG-c-Raf or the FLAG-tagged chimeric c-Raf/PKC construct, were treated with UV irradiation, serum, or PMA 2 days post-transfection. The data in Fig. 3 demonstrate that swapping the CRDs inhibited c-Raf activity in response to UV by 76% (p < 0.1, n = 3) and serum by 61% (p < 0.1, n = 3). In contrast, the PMA response was augmented 2-fold for the chimeric compared with wild type c-Raf (p = 0.02, n = 3). The inability of the chimera to respond to UV irradiation suggested that the c-Raf CRD was essential for c-Raf function not only in mediating the important Ras/c-Raf interaction but also as primary sensor of UV signal transmission. On the other hand, the enhanced responsiveness to PMA may be explained by Ras-independent c-Raf activation due to the fact that PMA binding to the CRD facilitated localization at the plasma membrane (55), in analogy to the well studied paradigm governing PKC cytosol-to-membrane translocation and activation (23).

**Fig. 5.** Retinol and AR are functional antagonists. A, retinoid-depleted FLAG-c-Raf-transfected COS cells were preincubated in the presence or absence of 1 μM AR for 15 min, followed by the addition of 1 μM retinol for 30 min, or left retinoid-free. The cells were UV-irradiated, and 10 min later, c-Raf activity was determined in anti-FLAG-c-Raf immunoprecipitate/kinase assays. PMA was used as positive control at 100 ng/ml. Transfection efficiency was determined by c-Raf Western blot. B, means ± S.E. of densitometric determinations of c-Raf kinase activities normalized for the amount of FLAG-c-Raf protein expression (n = 2).

**Fig. 6.** UV activation of c-Raf is inhibited but not reversed by NAC. The UV activation of c-Raf was inhibited by 1 mM NAC when added at the time of activation (lanes 3 and 5) but not when added to the fully active kinase for 1 min, as demonstrated by the addition of NAC 10 or 15 min postactivation (lanes 6 and 7, respectively). At 10 min, c-Raf reached maximal kinase function as demonstrated in kinetic studies (41).

**Fig. 7.** UV irradiation has a direct effect on c-Raf in vitro and in vivo. A, the direct effect of UV and H₂O₂ on c-Raf CRD was investigated in vivo using AMS as thiol-trapping probe (47). The effect of AMS conjugation on CRD was analyzed by Western blot on a 16.5% SDS-PAGE under nonreducing conditions. B, UV-induced release of Zn²⁺ from the c-Raf CRD was monitored by changes in TSQ fluorescence in vitro. UV irradiation caused Zn²⁺ release, and this release (dotted line) was enhanced in the presence of retinol (thick short-dashed line) and inhibited in the presence of AR (thick dashed and dotted line). The retinol-mediated Zn²⁺ release was antagonized by AR (solid line).
UV Activation of c-Raf Is Dependent on Ras/c-Raf Interaction—Growth factor-mediated activation of c-Raf is dependent on Ras/c-Raf interaction (16). It was therefore of interest to determine whether c-Raf activation by UV also required interaction with Ras. To address this question, the N17-Ras mutant (56) was used as a dominant negative element in FLAG-c-Raf cotransfection experiments in COS-7 cells. Fig. 9A shows the level of c-Raf kinase activity stimulated by UV in comparison with serum and PMA. The presence of the N17-Ras dominant-negative mutant caused a 66% reduction in the UV-mediated c-Raf activation (p = 0.007, n = 3), suggesting mandatory interaction with GTP-Ras, like receptor protein-tyrosine kinase-mediated c-Raf activation. Inhibition by 38% (p = 0.09, n = 3) of PMA-induced c-Raf activation in the presence of N17-Ras indicated the existence of a Ras-independent pathway, as previously documented by Marais et al. (57). UV activation, however, followed the classical, Ras-dependent pathway.

This conclusion was confirmed independently using a pharmacological inhibitor of Ras post-translational modification. Perillic acid inhibits cysteine isoprenylation, without which Ras cannot localize to the plasma membrane and remains inactive (58). Pretreatment of COS-7 cells for 12 h with 3 mM perillic acid resulted in 80% inhibition of UV-mediated FLAG-c-Raf kinase activation (Fig. 9B). These results are in agreement with those obtained by the Ras dominant-negative mutant. Taken together, they indicate that the Ras/c-Raf interaction was absolutely required not only for activation of c-Raf by growth factor but also by UV irradiation.

Tyrosine Phosphorylation and Serine or Threonine Dephosphorylation Are Essential for c-Raf Optimal UV Activation—Phosphorylation of tyrosine residues Tyr-340 and Tyr-341 is essential for c-Raf kinase function. To determine whether c-Raf activation by UV also required serine/threonine phosphorylation, COS-7 cells transfected with FLAG-c-Raf were treated in the absence or presence of 3 μM herbimycin A, 100 μM okadaic acid, or 1 mM vanadate for 30 min prior to stimulation by UV irradiation and serum (5% fetal calf serum). The activity of FLAG-c-Raf was determined by immunoprecipitate/kinase assays and Western blotting.

FIG. 8. The c-Raf CRD is essential for kinase function. FLAG-c-Raf wild type and FLAG-c-Raf chimera with the c-Raf CRD substituted for the PKCα C1B domain were activated by UV irradiation, serum (5% fetal calf serum), or phorbol ester (100 ng/ml PMA). The wild type responded to the stimuli, but the chimera, being unable to interact with Ras, did not respond to UV or serum. Ras-independent membrane targeting and activation may explain the effect of PMA on the chimera (55).

FIG. 9. UV activation of c-Raf is Ras-dependent. The N17-Ras dominant-negative mutant was overexpressed (A). Alternatively, the endogenous Ras molecule was disabled by treatment with 3 mM perillic acid in FLAG-c-Raf transfected COS cells (B). The cells were activated by UV, UV plus 1 μM retinol, serum (5% fetal calf serum), or PMA (100 ng/ml), and the activity of FLAG-c-Raf was determined by immunoprecipitate/kinase assays. For further details, see the legend to Fig. 4.

FIG. 10. Tyrosine phosphorylation and serine/threonine dephosphorylation are necessary for the optimal activation of c-Raf by UV irradiation. COS cells transfected with FLAG-c-Raf were treated in the absence or presence of 3 μM herbimycin A, 100 μM okadaic acid, or 1 mM vanadate for 30 min prior to stimulation by UV irradiation. The presence of okadaic acid caused a 67% decrease in UV-mediated c-Raf activation (p = 0.01, n = 3) (Fig. 10), compared with lane 2 with lane 6; okadaic acid alone caused a negligible increase in c-Raf basal activity (data not shown). These data indicate the importance of an okadaic acid-sensitive phosphatase, most likely protein phosphatase 2A, since this has been shown to form complexes with c-Raf in vitro and in vivo (18) during the activation of c-Raf kinase. Taken together, the data indicate that the direct chemical effect of UV irradiation on Ras/c-Raf interaction (16) during the activation of c-Raf kinase. Taken together, the data indicate that the direct chemical effect of UV irradiation...
tion on the molecule was not sufficient for c-Raf activation and did not bypass the requirements for Ras as well as tyrosine kinases and phosphatases, well known for their obligatory roles in growth factor and cytokine-mediated activation.

**DISCUSSION**

To understand the role of retinoids as regulators of c-Raf function, we pursued four seemingly separate lines of experimentation. When integrated, however, our results offer a new perspective on the function of the CRD, a subdomain of the N-terminal regulatory domain that was long suspected to play a crucial role in kinase activation. First, our previous finding that retinol bound the CRD was expanded to 14HRR, RA, and AR. These retinoids bound at the same site as retinol with nanomolar affinity, precisely replicating the findings with cer-

It has long been recognized that UV irradiation and the ROS this engenders lead to activation of a number of signal pathways (32, 36, 72). Prominent among these is the c-Raf/ MAPK axis that plays a role in physiological responses to UVB, for instance in skin cells (73–76). We used UVB irradiation of cells as a quasipharmacological, convenient mode of activation, since production of the actual mediator of kinase activation, ROS, is restricted to the light period. The consequences for c-Raf were then determined free of concern that continued exposure to ROS might accumulate damage, as might be the case with most oxidizing chemicals. Brief irradiation of cells initiated events that peaked 10 min later in the expression of substantial c-Raf phosphotransferase activity, determined in immunoprecipitates by phosphorylation of the disabled substrate, MEK. The presence of retinol promoted a substantial increase in kinase activity, confirming previous findings that peroxide-mediated activation of c-Raf and PKC was facilitated by bound retinol (41, 42). Extending the studies to natural retinoid metabolites, it was observed that 14HRR, as predicted from its agonistic properties in cell survival assays (50, 70), also enhanced UV-triggered c-Raf kinase activation, whereas AR did not (37–40, 69). The latter effect mirrored the physiological consequence of growth inhibition and apoptosis that cells experience when exposed to AR. We have provided evidence elsewhere that AR displaced retinol from the common receptors and thus mimicked a state of retinol deficiency, leading to growth retardation and apoptosis (37–40, 69). Similar physiological effects are not uncommon with cultures depleted of vitamin A (38, 50, 70). That retinol and AR acted as pharmacologic mutual inhibitors was also evident in the UV activation experiments (Fig. 5).

Since UV irradiation led to production of ROS, held responsible for the initiation of the c-Raf activation cycle, it was desirable to show direct chemical modifications of c-Raf. This was all the more necessary, since ROS reportedly activates protein-tyrosine kinase receptors (77–81), tyrosine kinases (14, 82, 83), and Ras (84, 85), but inactivates phosphatases (66, 86). These signal transduction molecules act to different degrees as upstream modulators of c-Raf, confounding the issue. However, the following observations indicate that c-Raf was indeed a direct target of ROS. First, ROS scavengers prevented c-Raf activation (Fig. 6). Second, analysis of the thiol content indicated distinct changes after UV irradiation of cells (Fig. 7A). The lowest number of thiol groups was found in peroxide treated cells, under conditions that promoted kinase activation. UV-induced changes could not be further assigned to specific cysteines due to the complexity of the molecule. Third, oxidation of cysteines in the CRD appeared the most likely scenario, in analogy to other CRD-containing enzymes, such as PKC α, β2, ε, and ζ (43), and the bacterial chaperone Hsp33 (48, 87). Oxidizing even one cysteine residue ought to compromise the chelation of Zn\(^{2+}\). Indeed, both Hsp33 and PKC α set free their Zn\(^{2+}\) ions under oxidizing conditions, and so did c-Raf (see below).

In the case of the Hsp33 chaperone, increased enzymatic capacity was directly attributed to a controlled unfolding of the molecules, and since this process was reversible by reduction, it was proposed that zinc fingers served as redox-sensitive hinges (48). Activated c-Raf did not revert to the inactive form by reduction with N-acetylcysteine, in contrast to PKC, where repeated oxidation/reduction cycles turned kinase activity on or off (42). The c-Raf CRD might therefore not obey the same paradigm of a reversible redox switch. On the other hand, early events in the activation cycle might depend on oxidative opening of the molecule, whereas the secondary modifications that c-Raf experiences by interaction with GTP/Ras might lock the kinase into an active conformation inured to reducing conditions.

Using the reductionist approach, we determined that ROS, generated by UV irradiation in vitro under the same conditions as in vivo, was capable of causing the rapid release of Zn\(^{2+}\) ions from the GST-c-Raf CRD fusion protein. We quote this observation as the fourth argument for direct effects on c-Raf. Although GST fusion proteins were not ideal tools to study protein function because of an inherent uncertainty of how well bacteria mastered folding mammalian peptides into their proper configuration, our results represent a credible in vitro correlate to the functional activation studies in vivo. This correlation was strengthened by the finding that GST-Raf fusion protein permitted substantially higher Zn\(^{2+}\) ion release when loaded with equimolar retinol. Conversely, AR suppressed Zn\(^{2+}\) release, whereas the equimolar mixture of retinol and AR behaved like retinoid-free protein (Fig. 7A). Thus, the biology of retinoids was mirrored by their biophysical effects on the isolated CRD.

The importance of the CRD for regulation of c-Raf kinase has been widely documented. Major contact sites enabling communication with GTP/Ras are embedded in this domain. The classical pathway, initiated by receptor phosphotyrosine kinases, is dependent on complex formation with Ras at the membrane. The alternate, redox-dependent pathway follows a similar route. First, redox activation of c-Raf required the presence of a CRD competent in Ras recognition. Substituting the PKC α CIB CRD for the c-Raf CRD abolished its activation by UV irradiation but shunted this chimeric kinase to the PKC pathway, similar to results described by Avruch and colleagues (55), since it became highly responsive to phosphol ester (Fig. 8). Second, competitive inhibition of the Raf/Ras interaction by overexpression of mutant N17-Ras was another indication of dependence on Ras. After modification by ROS, generated during UV irradiation, presumably involving oxidation of cysteine residues of the CRD, relocation of Zn\(^{2+}\) ions, and consequent conformational change, the interaction with GTP/Ras was still required. Furthermore, preventing prenylation and insertion of
Ras into the membrane curtailed UV activation of c-Raf.

Our studies re-emphasize the importance of agonistic retinoids for controlled activation of the c-Raf/MAPK by oxidizing agents. They also illustrate the role of zinc as an essential dynamic component of c-Raf. Both, control by retinol and zinc, converge on the same domain in the kinase. It is interesting to note that nutritional vitamin A and zinc deficiency produce many of the same symptoms: night blindness, sterility, defective wound healing, and abnormal skin regeneration. More research is needed to understand whether the underlying common parameter involves the CRD of c-Raf and related serine/threonine kinases. In distinction from its role as catalytic center in numerous enzymes, zinc occurs as a structural component in dozens of cytoplasmic and nuclear proteins including, notably, signal transduction molecules and transcription factors. Although “zinc fingers” are commonly thought of as rigid structures that enable in the case of transcription factors the intercalation into the DNA double helix, their role in the newly emerging redox regulation is apt to change our perception. The zinc fingers should be viewed as redox-regulated, dynamic, and reversible hinges, as proposed for the bacterial chaperone, Hsp33. Another view is that they act as redox sensors with provisions in mammalian cells for fine-tuning by different retinoids. Such a control element would allow c-Raf and its homologues to make constant adjustments in enzymatic output, as dictated by the changing redox status of cells.

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