Title: Activation of cRaf kinase by ultraviolet light: regulation by retinoids.

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Summary

The present study highlights retinoids as modulators of cRaf kinase activation by ultraviolet light (UV). While a number of retinoids, including retinol, 14-hydroxy-retro-retinol (14HRR), anhydroretinol (AR) and retinoic acid (RA) bound the cRaf 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 14HRR 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 cRaf activation, causing the release of Zn 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 cRaf kinase activation. Oxidation of cRaf 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 cRaf kinase competence. Thus, retinoids bound cRaf with high affinity, priming the molecule for UV/ROS mediated changes of the CRD that set off GTP-Ras interaction and, in context with an appropriate phosphorylation pattern, lead to full phosphotransferase capacity.
Introduction

The cRaf proto-oncogene is essential for cell growth, differentiation, and survival. Its major downstream effector is the mitogen-activated protein kinase (MAPK) (1-2) that elicits a complex set of cytosolic (3-5) as well as nuclear signals (6-9). The molecular mechanism of cRaf activation has not yet been fully elucidated (for review see Morrison 10-11). That growth factors and cytokines, as well as UV and ionizing radiation, all lead to the activation of the cRaf/MAPK pathway has been amply demonstrated (12-15). Receptor protein tyrosine kinase (RPTK), ligated by their respective growth factors, dimerise, 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 cRaf to translocate from the cytosol to the plasma membrane where an as yet unidentified mechanism bestows competence on cRaf to activate the MAPK cascade (16). Phosphorylation of tyrosine residues, for example Y340 and 341 (17) as well as dephosphorylation of serine residues S259, 621 (18) are believed to lock cRaf into the optimally competent form.

For docking with Ras two important contact sites in the regulatory domain of cRaf have been identified, one centered on the stretch of amino acids 51 to 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 cRaf 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 phosphatidylserine enhanced PKC activity
Several groups have identified lipid binding sites in the regulatory and catalytic regions of cRaf (27-30). Interestingly, Romero and colleagues suggested that phosphatidic acid mediated cRaf translocation from cytoplasm to membrane independently of its association with Ras (30). Besides the classical RTPK signal chain, alternative activation signals exist. Ultraviolet light and ionizing irradiation, as well as oxidizing agents, lead to cRaf/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 (ROS) is produced in every cell type by mitochondria as well as by dedicated enzyme systems. The changing view is that ROS, like nitric oxide (NO), 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).

While the chemistry of oxidative activation of serine/threonine kinases is poorly understood, it stands to reason that ROS target the most susceptible groups in cRaf, 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 was 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 cRaf 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 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 have recently been shown to shed their bound Zn$^{2+}$ ions as a result of oxidation (43, I. Korichneva, unpublished data). We therefore asked whether Zn$^{2+}$ ions would be liberated from the cRaf CRD during UV irradiation and whether pro-oxidant retinoids would impact on zinc release.

We further investigated whether UV mediated oxidation of the CRD was per se sufficient for kinase activation, but found that, unlike PKC alpha and zeta isoforms (42-43), cRaf required the additional productive interaction with GTP-bound Ras, as well as modifications in the tyrosine and serine/threonine phosphorylation patterns. Nevertheless, the CRD emerged as the key target structure for ROS elicited by stress-related mechanisms. Several findings in this report will highlight our conclusion: the triggering of cRaf activity by ROS generated during UV irradiation in vitro; the binding of pro-oxidant retinoids to the CRD; the conversion of thiol groups to disulfide by UV exposure in vivo; the release of Zn$^{2+}$ ions after UV irradiation; the requirement for the CRD to communicate with Ras. Therefore, despite its appearance as a rigid structural element, the CRD should be more appropriately viewed as a dynamic, redox-sensitive hinge with profound regulatory importance for initiation of the cRaf activation cycle by UV light.
Methods

Retinoids: All-trans isomers of AR and 14HRR were synthesized as described previously (37, 44). Trans-retinol, and RA were purchased from Sigma (St. Louis, MO) and purified by high pressure liquid chromatography. $^3$H-retinol was purchased from Dupont/NEN Life Science Products (Boston, MA).

Immunological reagents and chemicals: Anti-Flag® M2-Agarose affinity gel, herbimycin A, okadaic acid, vanadate, and perillic acid were obtained from Sigma (St. Louis, MO). Rabbit antibody to the cRaf C20-terminal peptide was purchased from Santa Cruz Biotechnology, Inc. (San Diego, Ca), N-(6-methoxy-8-quinolyl)-p-toluenesulfonamide (TSQ), and 4-acetamido-4'-maleimidylstilbene 2,2'-disulfonic acid (AMS) were obtained from Molecular Probes (Eugene, OR). Digitonin was bought from Fisher Scientific (Fairlawn, NJ).

Plasmids: Flag-cRaf was a gift from Dr. Roger J. Davis (University of Massachusetts, Worcester, MA). N17-Ras was obtained from Dr. N. M. Nathanson (University of Washington, Seattle, Washington). GST and Flag human cRaf CRD (136-LTTHNFARKTLAFCD ICQKFLLLNGFRCQTCGYKFHEHCSTKVPVCVDWSNIRQLL-195; this peptide contains the natural Trp-186 residue, responsible for fluorescence emission) were constructed as previously described (41). The Flag-cRaf chimera was constructed by replacing the cRaf CRD with the PKCα CRD. The following primers were used to isolate the PKCα CRD cDNA by polymerase chain reaction (Template kindly provided by Dr. T. Powell, Memorial Sloan-Kettering Cancer Center, NY, NY): 5 CAT GTT CCC CTC ACA ACA CAC AAG TTC AAA ATC CAC, and 5 GAT GTT ACT CCA GTC CAC GCA GAG GCT GGG GAC ATT GAT.
This product was used to synthesize the N- and C-termini of cRaf chimeric gene using the following primers: 5’ GCG GCC GCG AAT TCA ATG GAG CAC ATA CAG, and 5’TGA AGA CAG GTG GGA TCC TTA CTA GAA GAC AGG CAG CCT, respectively. The two products were used to generate the full-length molecule by PCR, which was then cloned into the EcoRI/BamH1 sites of the Sigma vector pFlag-CMV2. Fidelity was confirmed by sequencing.

**Transfection and UV activation:** Cos-7 cells were transfected according to the calcium phosphate method (45). Briefly: 5x10^5 cells were plated in 60 mm dishes the day before transfection. Two hours prior to transfection, the medium was replaced with fresh growth medium (Dulbecco’s modified Eagles’s medium high glucose {[DME-HG]} supplemented with 10% fetal calf serum). 12 µg DNA per dish were mixed with CaCl_2 and phosphate buffer to form a fine precipitate, which was then dispersed over the cells. The day after transfection the medium was removed and the cells washed twice with 3 ml each phosphate saline buffer (PBS). The cells were cultured with 2 ml of retinoid-free, phenol red-free DME-HG for 2 1/2 days prior to activation. UV-irradiation was performed for 2 min at 400 mW/cm² using 312 nm wavelength. Cultures were incubated at 37°C for 10 min after irradiation and harvested.

**cRaf immunoprecipitation/kinase assay:** Cells were lysed in 100λ 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 PMSF, and 1 mM vanadate. The lysates were precleared with 30λ of a 50% (vol/vol) protein G-agarose slurry, and the Flag-cRaf protein precipitated using 30λ of anti-Flag M2 affinity gel (Sigma). The immunoprecipitates were washed four times with lysis buffer containing 0.5 M NaCl, and twice with kinase buffer:
30 mM Tris-HCl, pH 7.5, 10 mM MgCl$_2$, 0.5 mM EGTA, and 1 mM vanadate. The kinase reaction was performed in 20λ kinase buffer using 200 ng of kinase-disabled His-MEK (K97M) (provided by Dr. R. Kolesnick, Memorial Sloan-Kettering Cancer Center) as substrate, 60 mM ATP, and 10 mCi $^{32}$P-$\gamma$-ATP (6,000 Ci/mmol). The reaction was carried out for 20 min at 30°C, and terminated by the addition of 10λ 4X Laemmli buffer (46). After separation by SDS PAGE on 7.5 % gels the proteins were transferred to PVD membrane, subjected to autoradiography and followed by Western analysis. Both Western blot and autoradiographs were evaluated by densitometry (BioRad GS 700 Densitometer with Quantity One software). Kinase activity values were normalized for the amount of Flag-cRaf precipitated. Data were analyzed by student T test.

**AMS trapping of free thiols in vivo:** Cos cells transfected with Flag-cRaf CRD were UV irradiated, treated with 1 or 10 mM H$_2$O$_2$, or left untreated, and incubated at 37°C for 10 min. The medium was removed, cells were washed with PBS, and 150λ of 100 mM iodoacetamide in permeabilization buffer (lysis buffer containing 20 µg/ml digitonin instead of 1% Triton X-100) (47) were 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-cRaf CRD protein was immunoprecipitated using anti-Flag M2 affinity gel as described above. The beads were resuspended in 25λ of 10 mM DTT, 100 mM Tris pH 8, 0.5% SDS, and incubated at 42°C for 1 h. Following reduction, 25λ of 50 mM AMS, 100 mM Tris pH 8, 0.5% SDS, were added and thiol-trapping allowed for 30 min at 37°C (48). The reaction was terminated by the addition of 10λ 4X non-reducing Laemmli buffer and boiling for 5 min. The samples were applied to a
16.5% Tris-Tricine SDS-PAGE gel from BioRad (Richmond, Ca.) and analyzed by Western blot using monoclonal anti-Flag M2 antibody (Sigma, St. Louis, MO).

**Bacteria growth and protein purification:** The cRaf-CRD was expressed as glutathione S-transferase (GST) fusion protein in the BL21/DE3 strain of *Escherichia coli* (Novagen). The growth conditions were as follows: the bacteria were initially grown at 37°C to an optical density at 600 nm of 0.5, then transferred to room temperature. When the optical density reached 0.7-0.8, protein synthesis was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside, and the cells harvested 2 h later. Bacteria were lysed by two passages through a French Press, and the proteins purified by affinity chromatography on the glutathione-agarose matrix (Sigma) according to a standard protocol. Purity by Coomassie blue staining of SDS-PAGE was usually >90% by this protocol.

**Retinoid binding assay:** The fluorescence emission spectra of 250 nM of GST-cRaf CRD fusion protein, equimolar concentrations of protein-retinoid complexes, as well as quantitative fluorescence measurement of 250 nM protein with retinoid titration at 25 nM increments, from a 75 µM stock solution in methanol, were performed in PBS, purged of oxygen by sparging with helium for 15 min, in a JASCO Spectrofluorimeter (model FP777), as described in detail (41). The protein solution was excited at 280 nm and the emission spectra monitored from 300 to 550 nm, or at 330 nm for the single wavelength measurements. All measurements were repeated 4 to 6 times. Binding constants were calculated by nonlinear curve fitting according to the theorem by Norris et al. (49). Complimentary binding data were obtained by the method of enhancement of retinol fluorescence (41).

**In vivo retinol binding assays:** Cos-7 cells were transfected with Flag-cRaf cDNA and cultured
in retinol-free medium as indicated above. Cells were preincubated with 100 nM retinol, 14HRR, AR, RA, or PS for 15 min prior to the addition of 5 nM $^3$H-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 PMSF, and 1 mM vanadate. cRaf immunoprecipitates were washed extensively with PBS containing 0.5 M NaCl and 0.5% bovine albumin, and incorporated counts measured by liquid scintillation counting. Results were expressed as differentials between immunoprecipitates of transfected and nontransfected cultures.

Zn release assay: A solution of 250 nM of GST-cRaf CRD fusion protein in PBS was UV irradiated at 312 nm for 2 min in the absence or presence of equimolar concentration of retinol, AR, or retinol + AR, followed by the addition of 3 µM TSQ. The changes in the fluorescence emission spectrum of TSQ (excitation $\lambda_{max}$ 335 nm) were monitored from 390-600 nm.

Results

Retinoids bind the cRaf CRD in vitro.

Two spectrofluorimetric methods, state of the art in the field (41, 49), were employed to measure binding of retinoids to the cRaf CRD 1. Quenching, based on the decrease in the intrinsic protein fluorescence due to resonance energy transfer (FRET) to a suitable bound ligand. Fluorescence emanated from the natural Trp-186 residue. Preliminary evidence (Hoyos, unpublished observation) suggested that retinol bound nearby at the second zinc-chelation center formed by Cys residues 165, 168 and 184, in cooperation with His-139. 2. Enhancement, or increase in the
intrinsic fluorescence of the ligand/retinoids, 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 cRaf and certain PKC CRDs with nanomolar affinity (41). To test for binding of other natural retinol metabolites, solutions of bacterially-expressed GST-cRaf-CRD fusion protein were excited at 280 nm, and the changes in protein fluorescence emission spectra were recorded, brought about by additions of stoichiometric amounts of retinoids. As illustrated in Fig. 1A, the fluorescence intensity of cRaf 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 to retinol, no detectable FRET signal was generated by the last three retinoids, probably owing to their poor fluorescence properties.

Binding of the retinoids, retinol, 14HRR, and AR, to the cRaf 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 equimolar amount of cRaf 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). cRaf CRD (250 nM) was excited at 280 nm and its fluorescence emission monitored at 330 nm after 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 non-linear curve fitting theorem developed by Norris et al (49), the apparent
dissociation constants in the nanomolar range were computed (Table 1). 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 cRaf in vivo.**

Retinol has been shown to bind cRaf in vivo (41). To demonstrate binding of other retinoids as well, a competition assay was developed, using Cos7 cell transfectants expressing the full length Flag-cRaf protein. Cells were labeled with 5nM 3H-retinol in the presence or absence of 1x10⁻⁷ M Rol, 14HRR, AR, and RA as cold competitors, and bound radioactivity was measured in cRaf immunoprecipitates using anti Flag antibody. All retinoids tested competed for binding of 3H-retinol to cRaf, demonstrating specific binding (Fig. 3). Phosphatidylserine (PS), a known ligand of the cRaf CRD, competed only moderately with retinol, indicating minor overlap of binding sites (27-29). Thus, the in vivo and in vitro binding results established that the cRaf CRD harbors a receptor site with equal specificity for four retinoids tested, extending our previous findings (41-42).

**Retinoids modulate cRaf activation by UV.**

Having demonstrated binding of retinoids to the CRD of cRaf in vitro and in vivo, it was important to inquire into the functional significance. Because UV-irradiation activated cRaf kinase capacity by an oxidative mechanism, and because retinol had emerged as pro-oxidant regulator (41-42), we tested whether retinoids served as cofactors in UV activation, using Flag-cRaf expressed to high level in Cos7 cells. The results of cRaf immunoprecipitation/kinase assays showed that retinoids indeed modulated the UV responses in dose-dependent fashion,
affecting the magnitude of kinase activation (Fig. 4), but not the kinetics (as published previously, 41). When compared to retinoid-deprived cultures, retinol and 14HRR at 1µM were found to produce an increase in phosphotransfer to disabled MEK, which peaked at 10 min (3.7 fold increase p=0.004, n=4; and 2.3 fold increase p=0.085, n=3, respectively). In contrast, AR and RA at 1µM produced decreases in Flag-cRaf kinase activity (1.8 fold decrease p=0.019, n=3; and 13.6 fold decrease p=0.003, n=3, respectively). On their own, retinoids had no discernible effect on kinase activity. Thus, the retinol metabolites tested showed opposite effects on cRaf kinase activation mediated by UV-irradiation: retinol and 14HRR enhanced, while RA and AR antagonized the activity. Vitamin E, a known anti-oxidant, had no effect on UV-mediated cRaf kinase activity (data not shown).

The retinoids are functional antagonists.

Retinoids bound the cRaf CRD equally well in vitro, competed for binding in vivo, but had opposite effects on survival of cells (38-40, 50). Because these effects were at least partially attributable to cRaf kinase regulation in vivo (41), we tested whether retinol and AR would behave as functional antagonists in cRaf kinase assays. As shown in Fig. 5 the inhibitory effects of AR on retinol, when used at equimolar concentrations, was evident. These results indicate that the retinoids compete for binding to cRaf, and act as functional antagonists.

UV activation of cRaf is not reversible by reduction.

UV-irradiation generates ROS (51-53). At low level 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 cRaf was abrogated by reducing agents. Fig. 6 illustrates 67% inhibition of cRaf activation by
the addition of L-N-acetyl cysteine (NAC) at the time of UV stimulation followed by incubated for 10 min (compare lane 3 to lane 5). However, once fully activated for 10 or 15 min cRaf could no longer be influenced by the addition of 1 mM NAC for 1 min (compare lane 3 to lane 6, and lane 4 to 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 cRaf in vivo via oxidation by ROS.**

To test whether ROS generated during UV stimulation had a direct effect on cRaf in vivo, we used the thiol-binding probe 4-acetamido-4'-maleidylstilbene-2, 2'-disulfonic acid (AMS) to test for the modification of thiol-groups (47, 54). This assay is predicated on the alkylation of free thiol groups, but not cystine 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 weight of the protein by 490 daltons. The altered size of Flag-cRaf CRD protein was assessed by electrophoresis in SDS PAGE under non-reducing conditions, although conformational changes could also contribute. The product of UV-irradiated cells migrated significantly slower, consistent with AMS modifications of the thiol-groups, compared to the reference protein from untreated cells (**Fig. 7A**). Oxidation of cells by two different concentrations of hydrogen peroxide caused the appearance of two differently migrating bands, the slower of which representing the higher degree of oxidation with the relatively larger number of disulfide groups that, after the in vitro reduction, were trapped by AMS.

**UV irradiation of cRaf CRD results in zinc release.**
The cRaf CRD comprises two zinc-coordinated centers, one of which is formed by the three thiol groups of C165, 168, and 184 and one imino group of H139, whereas the other C152, 155, 176 and H172. 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 cRaf CRD. Fig. 7B illustrates that the cRaf-CRD fusion protein indeed shed its zinc upon UV-irradiation, as determined by the binding of liberated zinc to N-(6-methoxy)-8-quinolyl-toluene sulfonamide (TSQ), a zinc-sensitive fluorescent probe, in agreement with recent in vivo observations (I. Korichneva, unpublished data). 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 Zn ions from the cRaf 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. 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 cRaf kinase.

The cRaf CRD is essential for cRaf function.

cRaf shares the structural CRD motif with members of the protein kinase C (PKC) family (21). Furthermore, the homologous domains in the PKC family bearing the diacyl glycerol 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 cRaf CRD domain with the PKCα C1B domain. Cos-7 cells, transfected with wild type Flag-cRaf or the Flag-tagged chimeric cRaf/PKC construct, were
treated with UV irradiation, serum, or PMA two days post-transfection. The data in Fig. 8 demonstrate that swapping the CRDs inhibited cRaf 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 to wild type cRaf (p=0.02, n=3). The inability of the chimera to respond to UV irradiation suggested that the cRaf CRD was essential for cRaf function not only in mediating the important Ras/cRaf 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 cRaf activation owing 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).

**UV activation of cRaf is dependent on Ras/cRaf interaction.**

Growth factor-mediated activation of cRaf is dependent on Ras/cRaf interaction (16). It was, therefore, of interest to determine whether cRaf activation by UV also required interaction with Ras. To address this question, the N17-Ras mutant (56) was used as dominant negative element in Flag-cRaf cotransfection experiments in Cos-7 cells. Fig. 9A shows the level of cRaf kinase activity stimulated by UV in comparison with serum, and PMA. The presence of the N17Ras dominant-negative mutant caused a 66% reduction in the UV-mediated cRaf activation (p=0.007, n=3), suggesting mandatory interaction with GTP-Ras, like RPTK-mediated cRaf activation. Inhibition by 38% (p=0.09, n=3) of PMA-induced cRaf activation in 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...
translation modification. Perillic acid inhibits cysteine isoprenylation without which Ras can not localize to the plasma membrane and remains inoperative (58). Pretreatment of Cos-7 cells for 12 h with 3 mM perillic acid resulted in 80% inhibition of UV mediated Flag-cRaf 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/cRaf interaction was absolutely required not only for activation of cRaf by growth factor, but also by UV irradiation.

**Tyrosine phosphorylation and serine or threonine dephosphorylation are essential for cRaf optimal UV activation.**

Phosphorylation of tyrosine residues Y340 and 341 are essential for cRaf activation, one of the kinases responsible being a member of the Src family (59-60). To evaluate the role of Src kinases in the cRaf activation by UV, herbimycin A- a potent Src inhibitor- was used (61-62). Cos-7 transfectants were treated with 3 µM herbimycin A for 30 min prior to stimulation. Fig. 10 shows that the activation of cRaf mediated by UV is decreased by 47% (p=0.1, n=2), and that of serum by 71% (p=0.09, n=2) (compare lane 2 to 4, and 3 to 5) in herbimycin A pre-treated cultures, indicating that UV stimulation does not bypass the requirement for cRaf tyrosine phosphorylation.

Tyrosine kinases are an essential component of cRaf activation. Omnipresent tyrosine phosphatases (PTP) are counteracting tyrosine phosphorylation. Hence, their inhibition may result in the constitutive activation of cRaf. As phosphatases are known to be deactivated by ROS (63, 65), as demonstrated especially for PTP 1B with its ROS-sensitive cysteine in the catalytic domain (66), it was necessary to demonstrate UV activation in cells in which PTPs were blocked by pharmacological inhibitors. Flag-cRaf transfected Cos-7 cells were therefore UV-
irradiated in the presence of 1mM sodium vanadate, a specific inhibitor of tyrosine phosphatases.

**Fig. 10** shows that UV irradiation caused a 20% increase in cRaf activation in vanadate treated cells (p=0.1, n=3) (compare lane 8 to lane 9); vanadate alone increased cRaf basal activity (compare lane 1 to lane 8). The fact that UV caused an additive effect over that of vanadate alone suggests that UV/ROS impacts on cRaf directly.

To determine the influence of serine/threonine phosphatases on cRaf activation, we treated Flag cRaf transfected Cos-7 cells with 100 nM okadaic acid (67-68) for 30 min prior to stimulation by UV. The presence of okadaic acid caused a 67% decrease in UV-mediated cRaf activation (p=0.01, n=3) (**Fig 10** compare lane 2 to 6); okadaic acid alone caused a negligible increase in cRaf basal activity (data not shown). These data indicate the importance of an okadaic acid-sensitive phosphatase, most likely PP2A, since this has been shown to form complexes with cRaf in *vitro* and *in vivo* (18) during the activation of cRaf kinase. Taken together, the data indicate that the direct chemical effect of UV-irradiation on the molecule was not sufficient for cRaf 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 cRaf 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 findings 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 certain PKC CRDs (42). While they share the same beta-ionone ring and polyene structure that presumably furnishes the contact surface, they differ in the functional groups at their tail ends. The presence or absence of hydroxyls dictated biological function, as retinol, and 14HRR acted as agonists, whereas AR that lacks hydroxyl groups was antagonistic (37-40, 50, 69-71). Complementing the in vitro binding studies (Figures 1, 2 and Table 1) we also furnish evidence for binding in intact cells, and provide at least qualitative evidence that all four had affinity for full-length cRaf in native conformation (Figure3). As retinol and 14HRR are nearly ubiquitous in tissues at concentrations (2x10^-6 and 1 x 10^-7 M, respectively) exceeding the Kd of the CRD/retinoid complex (2x10^8 Mol^-1) one can safely assume that inside cells cRaf is always loaded with retinoids.

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 cRaf/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 quasi-pharmacological, convenient mode of activation, since production of the actual mediator of kinase activation, ROS, is restricted to the light period. The consequences for cRaf 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 ten min later in the expression of substantial cRaf 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 results that peroxide-mediated activation of cRaf and PKC was facilitated by bound retinol (41-42). Extending the studies to natural retinol metabolites, it was
observed that 14HRR, as predicted from its agonistic properties in cell survival assays (50, 70), also enhanced UV-triggered cRaf 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 (Figure 5).

Since UV irradiation led to production of ROS, held responsible for the initiation of the cRaf activation cycle, it was desirable to show direct chemical modifications of cRaf. This was all the more necessary since ROS reportedly activates protein tyrosine kinase receptors (77-81), tyrosine kinases (14, 82-83), Ras (84-85), and to inactivate phosphatases (66, 86). These signal transduction molecules act to different degrees as upstream modulators of cRaf, confounding the issue. However, the following observations indicate that cRaf was indeed a direct target of ROS: First ROS scavengers prevented cRaf activation (Figure 6). Second, analysis of the thiol content indicated distinct changes after UV irradiation of cells (Figure 7A). The fewest 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 owing 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 alpha, beta 2, epsilon, and zeta (43), and the bacterial chaperone Hsp33 (48, 87). Oxidizing even one cysteine residue ought to compromise the chelation of Zn²⁺. Indeed, both Hsp33 and PKC alpha set free their Zn²⁺ ions.
under oxidizing conditions, and so did cRaf (vide infra).

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 cRaf did not revert to the inactive form by reduction with N-acetyl cysteine, in contrast to PKC where repeated oxidation/reduction cycles turned kinase activity on or off (42). The cRaf 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 cRaf 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 cRaf CRD fusion protein. We quote this observation as the fourth argument for direct effects on cRaf. 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 findings 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 (Figure 7A). Thus, the biology of retinoids was mirrored by their biophysical effects on the isolated CRD.
The importance of the CRD for regulation of cRaf 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 cRaf required the presence of a CRD competent in Ras recognition. Substituting the PKC alpha CIB CRD for the cRaf 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 phorbol ester (Fig 8). Second, competitive inhibition of the Raf/Ras interaction by over-expression of mutant N17-Ras was another indication for 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 cRaf.

Our studies re-emphasize the importance of agonistic retinoids for controlled activation of the cRaf/MAPK by oxidizing agents. They also illuminate the role of zinc as an essential dynamic component of cRaf. 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 cRaf and related serine/threonine kinases. In distinction to its role as catalytic center in numerous enzymes, zinc occurs as 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 cRaf and its homologues to make constant adjustments in enzymatic output, as dictated by the changing redox status of cells.

Acknowledgements

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The abbreviations used are: Rol, retinol; 14HRR, 14-hydroxy-retro-retinol; AR, anhydroretinol; RA, retinoic acid; CRD, cysteine rich domain; UV, ultraviolet light; ROS, reactive oxygen species; NO, nitric oxide; MAPK, mitogen activated protein kinase; PKC, protein kinase C; TSQ, N-(6-methoxy-8-quinolyl)-p-toluene sulfonamide; AMS, 4-acetamido-4-maleimidylstilbene-2,2-disulfonic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; GST, Glutathione-S-transferase; PBS, phosphate saline buffer; PS, phosphatidylserine; FRET, fluorescence resonance energy transfer; NAC, L-N-acetyl cysteine;
PTP, protein tyrosine phosphatase; Hsp33, heat shock protein 33; PA, parinaric acid; FCS, fetal calf serum; CMV-2, cytomegalovirus-2; PMA, phorbol 12-myristate 13-acetate.
Legends.

Figure 1A. Quenching of GST-cRaf CRD intrinsic fluorescence by retinoids. Fluorescence emission spectra of 250 nM solution of GST fusion protein comprising amino acids 136 to 195 of human cRaf excited at 280 nm in the absence (— —) or presence of 250 nM 14HRR (— —), AR (— - -), RA (-----) in phosphate buffered saline (PBS); and the spectra of free14HRR (— — —), AR (— - -), and RA (— —) in PBS.

Figure 1B. Enhancement of the fluorescence emission of retinoids bound to GST cRaf CRD. The fluorescence emission spectra of retinol (— —), 14HRR (— - -), and AR (— —) are shown in complex with stoichiometric amounts (250 nM) of the GST-cRaf CRD fusion protein, comprising amino acids 136-195, as well as the fusion protein alone (— - - - -), and free retinoids: retinol (— —), 14HRR (----), AR (— - -) in PBS. The excitations for retinol, 14HRR, and AR were 325, 348, and 368 nm, respectively.

Figure 2. Retinoid binding to the GST-cRaf CRD is quantitative. 250 nM solutions of GST-cRaf CRD in PBS were titrated with all-trans retinol (c), 14HRR (1), AR (ê), and parinaric acid (PA) (◊) used as negative control, added in 25 nM increments from a 75 µM stock solution in methanol. The protein solutions were excited at 280 nM and the changes in the protein’s fluorescence emission monitored at the protein $\lambda_{\text{max}}$ 330 nm. The fluorescence intensity reading were corrected for inner filtering and plotted versus retinoid concentrations.

Figure 3. Retinoids compete for binding to cRaf in vivo. Cos cells expressing Flag tagged cRaf were pretreated for 15 min with 100 nM of unlabeled retinol, 14HRR, AR, RA, or phosphatidylserine (PS), followed by incubation for 30 min with 5 nM $^3$H-retinol. Values for
incorporated counts of the immunoprecipitates, generated with agarose conjugated anti-FLAG antibody, were corrected for the radioactivity in control immunoprecipitates of non-transfected cells, (n=4).

**Figure 4.** *Retinoids regulate the UV activation of cRaf.* Retinoids-depleted Flag-cRaf transfected Cos cells were reconstituted with the indicated concentrations of retinoids for 30 min or left retinoid free. The cells were UV-irradiated and 10 min later cRaf activity was determined using disabled His-MEK as the substrate in anti-Flag cRaf immunoprecipitate/kinase assays. Transfection efficiency was determined by cRaf Western blot. **A:** Autoradiograph, and Western blot of samples treated with retinol. **B:** Autoradiograph and Western blot of cultures treated with 14HRR. **C:** Means ± SE and p-values of densitometric determinations of cRaf kinase activities normalized for amount of Flaf-cRaf protein, n=4 for Rol, and n=3 for 14HRR treated cells. **D:** Autoradiograph and Western blots of cultures treated with AR. **E:** Autoradiograph and Western blot of cultures treated with RA. **F:** Means ± SE and p-values of densitometric determinations of cRaf kinase activities normalized for amount of Flaf-cRaf protein, n=3 for AR and RA treated cultures (** compared to untreated cells; * compared to UV irradiated cells).**

**Figure 5.** *Retinol and AR are functional antagonists.* **A:** Retinoid-depleted Flag-cRaf 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 cRaf activity was determined in anti-Flag cRaf immunoprecipitate/kinase assays. PMA was used as positive control at 100 ng/ml. Transfection efficiency was determined by cRaf Western blot. **B:** Means ± SE of densitometric determinations of cRaf kinase activities normalized for the amount of Flaf-cRaf protein expression, n=2.
**Figure 6.** *UV activation of cRaf is inhibited but not reversed by NAC.* The UV activation of cRaf 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 one minute, as demonstrated by the addition of NAC 10 or 15 min post activation (lanes 6 and 7, respectively). At 10 min cRaf reached maximal kinase function as demonstrated in kinetic studies (41).

**Figure 7.** *UV-irradiation has a direct effect on cRaf in vitro and in vivo.* A: The direct effect of UV and H₂O₂ on cRaf 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 non-reducing conditions. B: UV induced release of Zn²⁺ from the cRaf CRD was monitored by changes in TSQ fluorescence in vitro. UV-irradiation caused Zn²⁺ release (-----) and this release was enhanced in the presence of retinol (- - - -), and inhibited in the presence of AR (-----). The retinol mediated Zn²⁺ release was antagonized by AR (-----).

**Figure 8.** *The cRaf CRD is essential for kinase function.* Flag-cRaf wild type and Flag-cRaf chimera with the cRaf CRD substituted for the PKCα C1B domain were activated by UV-irradiation, serum (5% FCS), 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).

**Figure 9.** *UV activation of cRaf is Ras-dependent:* The N17Ras dominant-negative mutant was over-expressed (A). Alternatively, the endogenous Ras molecule was disabled by treatment with 3mM perillic acid in Flag-cRaf transfected Cos cells (B). The cells were activated by UV, UV
+ 1 μM retinol, serum (5% FCS), or PMA (100 ng/ml) and the activity of Flag-cRaf determined by immunoprecipitate/kinase assays. For further detail see legend to Figure 4.

**Figure 10.** Tyrosine phosphorylation and serine/threonine dephosphorylation is necessary for the optimal activation of cRaf by UV-irradiation. Cos cells transfected with Flag-cRaf were treated in the absence or presence of 3 μM herbimycin A, 100 nM okadaic acid, or 1 mM vanadate for 30 min prior to stimulation by UV-irradiation, and serum (5% FCS). The activity of Flag-cRaf was determined by immunoprecipitate/kinase assays and Western blotting.
Table 1

Apparent dissociation constants \( \{ \text{nM} \pm \text{SE}, n=5 \} \) of cRaf CRD

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References.

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    22300-4
    Protein Sci. 6, 477-80
    Chem. 270, 14679-84
    J. Biol. Chem. 269, 10000-7
    271, 8472-80

31

32


Figure 1B
Figure 2
Figure 3

3H-Retinol Binding [%]

Competitor [10^{-7} M]

- None
- Retinol
- 14HRR
- AR
- RA
- PS

Flag-cRaf
Figure 4
Figure 5
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Figure 6
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Figure 9

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**Figure 10**
Activation of cRaf kinase by ultraviolet light: Regulation by retinoids
Beatrice Hoyos, Asiya Imam, Irina Korichneva, Ester Levi, Ramon Chua and Ulrich Hammerling

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