

- after transfection with NLthyΔBgl or NLthyΔBglVprX and pLET-LAI. Supernatant was collected 48 hours after transfection and concentrated by ultracentrifugation at 40,000g for 1 hour.
19. T. J. McQuade *et al.*, *Science* **247**, 454 (1990); T. D. Meek *et al.*, *Nature* **343**, 90 (1990); N. A. Roberts *et al.*, *Science* **248**, 358 (1990).
 20. Virus produced in the presence of PI was obtained after transfection with either NLthyΔBglVprX or NLthyΔBgl and pCMV-VSV-G. At 24 hours after transfection, the medium was replaced with medium containing 100 nM indinavir sulfate (Merck). Supernatant was collected 48 and 72 hours after transfection and concentrated (9).
 21. J. Kimpton and M. Emerman, *J. Virol.* **66**, 2232 (1992); S. P. Layne *et al.*, *Virology* **189**, 695 (1992).
 22. M. Piatak, Jr. *et al.*, *Science* **259**, 1749 (1993); T.-W. Chun *et al.*, *Nature* **387**, 183 (1997).
 23. V. Ayyavoo *et al.*, *Nature Med.* **3**, 1117 (1997).
 24. K. Tenner-Racz *et al.*, in *Progress in AIDS Pathology*, H. Rotterdam and S. C. Sommers, Eds. (Field and Wood, New York, 1989), pp. 29–40; J. Embretson *et al.*, *Nature* **362**, 359 (1993); G. Pantaleo *et al.*, *ibid.*, p. 355.
 25. G. M. Shearer *et al.*, *J. Immunol.* **137**, 2515 (1986); B. Wahren *et al.*, *J. Virol.* **61**, 2017 (1987).
 26. J. K. Wong *et al.*, *Science* **278**, 1291 (1997); D. Finzi *et al.*, *ibid.*, p. 1295; J. K. Wong *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 12574 (1997); T.-W. Chun *et al.*, *ibid.*, p. 13193.
 27. B. Autran *et al.*, *Science* **277**, 112 (1997); M. Connors *et al.*, *Nature Med.* **3**, 533 (1997).
 28. Concentrated virus was lysed in 2× loading buffer and subjected to electrophoresis on SDS–15% polyacrylamide gels (9). Western blotting was performed with a rabbit polyclonal antibody for Vpr (provided by N. Landau, Aaron Diamond AIDS Research Center, New York) or human anti-HIV hyperimmune plasma (provided by P. Krogstad and Y. Bryson, UCLA) and developed with the enhanced chemiluminescence assay (Amersham, Arlington Heights, IL).
 29. The method of double staining for surface marker Thy 1.2 and DNA content was performed as described in (9, 10). All stained cells were acquired on a FACScan II apparatus (Becton-Dickinson) and analyzed with Cell Quest software.
 30. We thank D. S. An, J. Zack, and P. Krogstad for valuable reagents and advice and Vaheideh Gudeman for technical support. Supported by NIH grant CA70018 and Center for AIDS Research grant AI28697. B.P. and K.G.-F. were supported by NIH postdoctoral training grant T32/A107388.

3 December 1997; accepted 3 June 1998

Specific Covalent Labeling of Recombinant Protein Molecules Inside Live Cells

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Recombinant proteins containing four cysteines at the *i*, *i* + 1, *i* + 4, and *i* + 5 positions of an α helix were fluorescently labeled in living cells by extracellular administration of 4',5'-bis(1,3,2-dithioarsolan-2-yl)fluorescein. This designed small ligand is membrane-permeant and nonfluorescent until it binds with high affinity and specificity to the tetracysteine domain. Such in situ labeling adds much less mass than does green fluorescent protein and offers greater versatility in attachment sites as well as potential spectroscopic and chemical properties. This system provides a recipe for slightly modifying a target protein so that it can be singled out from the many other proteins inside live cells and fluorescently stained by small nonfluorescent dye molecules added from outside the cells.

Attachment of fluorescent or other useful labels onto proteins has traditionally been accomplished by in vitro chemical modification after purification (1). Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* can be genetically fused with many host proteins to produce fluorescent chimeras in situ (2, 3). However, GFP is potentially perturbative because of its size (238 amino acids), can usually only be fused at the NH₂- or COOH-terminus of the host protein, offers a limited variety of colors, and is of no assistance for spectroscopic readouts other than fluorescence. We therefore designed and synthesized a tight-binding pair of molecular components: a small receptor domain composed of as few as six natural amino

acids that could be genetically incorporated into proteins of interest, and a small (<700-dalton), synthetic, membrane-permeant ligand that could be linked to various spectroscopic probes or crosslinks. The ligand has relatively few binding sites in nontransfected mammalian cells but binds to the designed peptide domain with a nanomolar or lower dissociation constant. An unexpected bonus is that the ligand is nonfluorescent until it binds its target, whereupon it becomes strongly fluorescent.

Our approach exploits the facile and reversible covalent bond formation between organoarsenicals and pairs of thiols. Trivalent arsenic compounds bind to the paired thiol groups of proteins containing closely spaced pairs of cysteines or the cofactor lipoic acid (4, 5). Such binding, which is responsible for much of the toxicity of arsenic compounds, is completely reversed by small vicinal dithiols such as 2,3-dimercaptopropanol [British anti-Lewisite (BAL)] or 1,2-ethanedithiol (EDT), which form tighter complexes with the organoarsenical than do cellular dithiols (6, 7). If a peptide domain could be designed with even higher affinity than that of the antidotes for an organoarsenical ligand, the ligand could be administered in the presence of excess antidote and specifically

bind the desired peptide domain without poisoning other proteins. To achieve this unusual affinity, we designed a peptide domain with four cysteines already organized to bind an organic molecule containing two appropriately spaced trivalent arsenics (Fig. 1). If the distance between the two pairs of cysteines matched the spacing between the arsenics, the two dithiol-arsenic interactions should be highly cooperative and entropically favorable. The four cysteines were placed at the *i*, *i* + 1, *i* + 4, and *i* + 5 positions of an α helix, so that the four thiol groups would form a parallelogram on one side of the helix. We chose acetyl-WEAAAREACCCECCARA-amide (8) as a model peptide for in vitro tests, on the basis of the known propensity of peptides of the form acetyl-W(EAAAR)_n-A-amide (9) to form α helices.

Fourteen biarsenical ligands were synthesized and tested for their ability to bind the

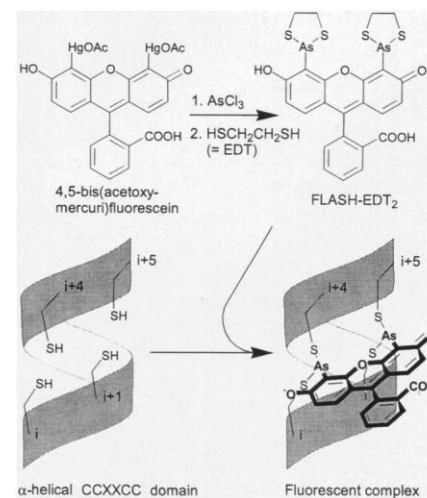


Fig. 1. Synthesis of FLASH (20) and proposed structure of its complex with an α -helical tetracysteine-containing peptide or protein domain. Although the structure is drawn with the *i* and *i* + 4 thiols bridged by one arsenic and the *i* + 1 and *i* + 5 thiols bridged by the other, we cannot rule out the isomeric complex in which one arsenic links the *i* and *i* + 1 thiols while the other links the *i* + 4 and *i* + 5 thiols.

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REPORTS

Fig. 2. Fluorescence of FLASH is induced by binding to a tetracysteine motif. **(A)** Fluorescence excitation (Exc) and emission (Em) spectra of 250 nM FLASH bound either to a model tetracysteine-containing peptide (20) in phosphate-buffered saline at pH 7.4 (solid lines) or to EDT at the same gain settings (dashed line, emission spectrum only). **(B)** Kinetics of binding of 1 μ M FLASH to 10 μ M peptide in the presence of 10 μ M EDT and subsequent reversal by a higher concentration (5 mM) of EDT. The apparent fluorescence of FLASH-EDT₂ ranged from 0.05% (A) to 0.5% (B) of that of the FLASH-peptide complex and might merely reflect trace impurities such as free fluorescein.

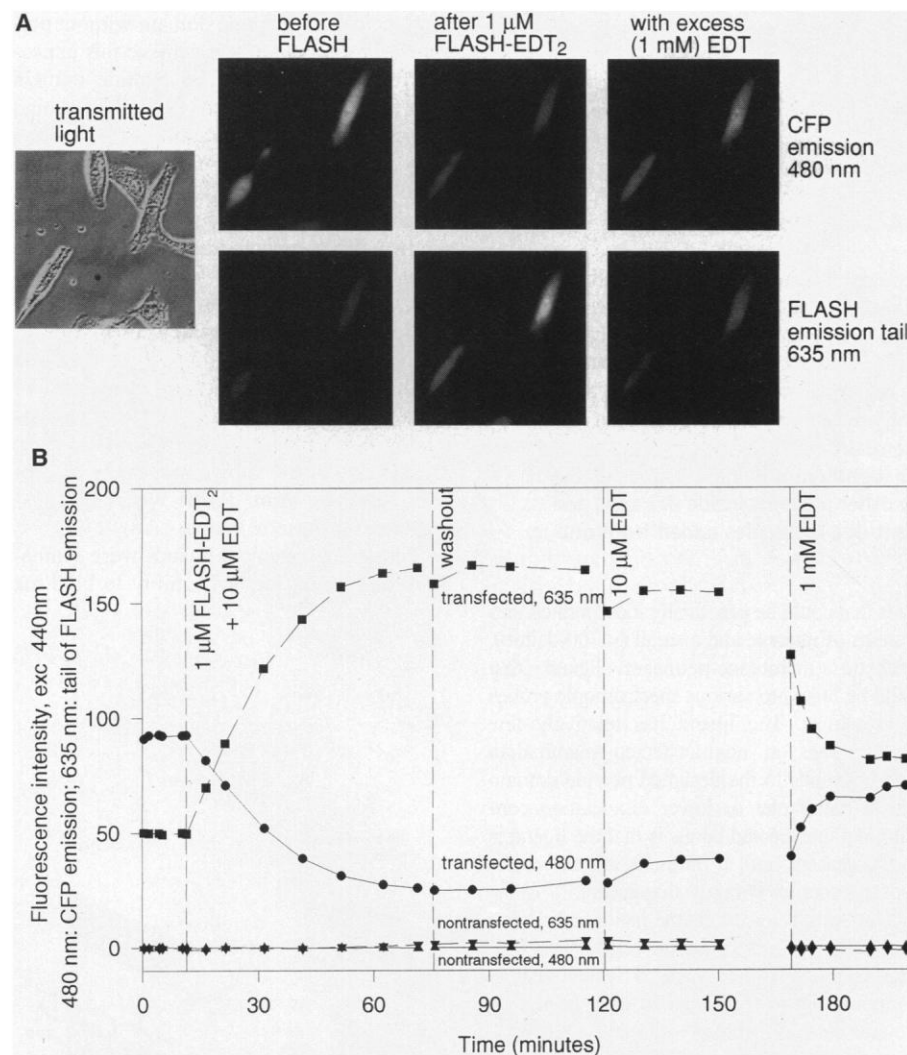
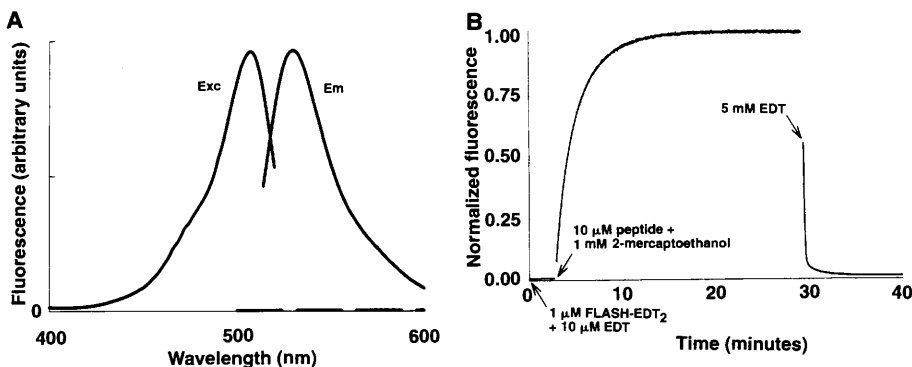


Fig. 3. FLASH labeling of a tetracysteine motif appended to a GFP mutant in living cells. **(A)** Reversible intracellular labeling of a tetracysteine-containing peptide, AEAAAREACRECCARA (8), fused to the COOH-terminus of ECFP (13, 14) and expressed in HeLa cells (21). Fluorescence images (22) were recorded before and after incubation with 1 μ M FLASH-EDT₂ and after treatment with 1 mM EDT. Excitation was at 440 ± 10 nm, and emissions were collected at 480 ± 15 nm (top row) and 635 ± 25 nm (bottom row). Comparison with a transmitted-light view (far left) shows that cells not expressing the fusion protein showed negligible fluorescence at either emission wavelength. Fluorescence images were taken at 4, 150, and 195 min. **(B)** Time course of intensities at cyan (circles) and red (squares) emission wavelengths for the fluorescent cell at the upper right, compared to the same measurements (up- and down-pointing triangles) on a nonexpressing cell. Excess FLASH and EDT were removed as indicated (washout), then the cells were incubated with the indicated concentrations of EDT.

tetracysteine peptide in the presence of a small excess of BAL or EDT (10), but the only successful ligand was 4',5'-bis(1,3,2-dithioarsolan-2-yl)fluorescein, which may also be called FLASH-EDT₂ [fluorescein arsenical helix binder, bis-EDT adduct (Fig. 1)]. FLASH-EDT₂ was prepared in a single step by transmetallation (11, 12) of commercially available fluorescein mercuric acetate, followed by addition of EDT to facilitate purification. The product was virtually nonfluorescent but became brightly fluorescent after addition of the tetracysteine peptide to displace the EDT. The excitation and emission peaks were 508 and 528 nm, respectively (Fig. 2), which are about 20 nm longer in wavelength than those of free fluorescein. The quantum yield of the FLASH-peptide complex was 0.49, whereas FLASH-EDT₂ was $\leq 5 \times 10^{-4}$ times as fluorescent. The small size of EDT probably permits rotation of the aryl-arsenic bond and excited state quenching by vibrational deactivation or photoinduced electron transfer, whereas the peptide complex may evade such quenching because its more rigid conformation should hinder conjugation of the arsenic lone pair electrons with the fluorescein orbitals. The equilibrium reaction $\text{FLASH-EDT}_2 + \text{peptide} \rightleftharpoons \text{FLASH-peptide} + 2 \text{EDT}$ favored FLASH-peptide at $\leq 10 \mu\text{M}$ EDT and FLASH-EDT₂ at $\geq 1 \text{ mM}$ EDT, so that labeling was reversed by millimolar concentrations of EDT (Fig. 2B). Monothiol such as 2-mercaptoethanol or glutathione were helpful to catalyze equilibration but did not compete themselves. The FLASH-peptide complex showed no sign of dissociation even when diluted to 1 nM in 5 mM 2-mercaptoethanol and left for weeks, which indicates that complex formation was essentially irreversible in the absence of excess EDT. The apparent pK_a of the fluorescein chromophore in the FLASH-peptide complex was 5.4, so the fluorescence should not be sensitive to variations in cytosolic pH near 7.

To test the membrane permeability of FLASH-EDT₂ and the specificity of the FLASH-peptide interaction in live mammalian cells, we genetically fused the designed peptide

REPORTS

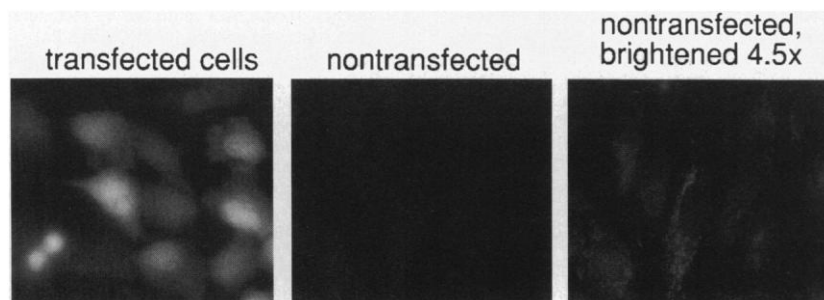


Fig. 4. FLASH labeling of a tetracysteine motif inserted within a protein. **(Left)** Fluorescence images (excitation 480 ± 15 nm, emission 535 ± 12.5 nm) of HeLa cells transiently transfected with a gene for Cys^{6,7,10,11}-calmodulin, labeled 36 hours later with 1 μ M FLASH-EDT₂ and 10 μ M EDT in Hank's balanced salt solution for 1 hour at 25°C, then washed free of excess dye just before imaging. **(Middle)** Nontransfected HeLa cells labeled and imaged under identical conditions. **(Right)** Nontransfected controls reimaged at 4.5-fold higher gain.

(with the tryptophan changed to an alanine) to the COOH-terminus of a cyan mutant [enhanced cyan fluorescent protein (ECFP)] of GFP (13, 14) and transiently expressed this fusion protein in HeLa cells. ECFP was chosen as the host protein so that expressing cells could be distinguished from nonexpressing cells by ECFP fluorescence (Fig. 3) and because formation of the ECFP-Cys₄ peptide-FLASH complex should be demonstrable by fluorescence resonance energy transfer (FRET) from ECFP to FLASH. Before addition of FLASH-EDT₂, some cells were brightly fluorescent at the ECFP emission maximum (480 nm) and only very dimly fluorescent at 635 nm, at which ECFP barely emits; whereas the other cells did not express ECFP. When 1 μ M FLASH-EDT₂ was added with 10 μ M EDT, cells already expressing ECFP increased their fluorescence at 635 nm more than threefold, because of the long-wavelength tail of FLASH emission, whereas the fluorescence of ECFP at 480 nm declined by >70%, indicating FRET from the ECFP to the bound FLASH. The high efficiency of FRET showed that >70% of the intracellular ECFP molecules had directly bound FLASH and that the distance between the chromophores was <5 nm as expected from the protein dimensions (15). Cells not expressing ECFP were unaffected by FLASH-EDT₂. After binding was complete, which required about 1 hour (Fig. 3B), removal of FLASH from the medium while maintaining 10 μ M EDT had little effect, but 1 mM EDT largely reversed the effects of FLASH-EDT₂ (Fig. 3, A and B). The controllable onset and reversibility of binding and labeling should prove valuable in many applications.

The tetracysteine motif also worked when introduced into an endogenous α helix. The gene for *Xenopus* calmodulin (14) was mutated to replace four residues of the NH₂-terminal α helix (Glu⁶, Glu⁷, Ala¹⁰, and Glu¹¹) with cysteines. The cytosol and nuclei of HeLa cells expressing this mutant calmodulin became brightly fluorescent when treated with 1 μ M FLASH-EDT₂ and 10 μ M EDT (Fig. 4). Con-

trol nontransfected cells in a different dish showed dimmer staining, which appeared to be mostly mitochondrial. This background labeling could be somewhat further reduced by higher EDT concentrations, which would not interfere with FLASH labeling if the affinity of the tetracysteine peptide for FLASH could be increased by combinatorial optimization of adjacent residues.

FLASH-EDT₂ (1 μ M) administered with 10 μ M EDT had no detectable effect for up to 4 hours on the viability of HeLa cells and Jurkat lymphocytes assayed by propidium iodide exclusion; reduction of methylthiazolyl-diphenyltetrazolium to the colored formazan (16); or ability to respond to muscarinic stimulation, assessed by activation of the nuclear factor of activated T cells and monitoring of the expression of a β -lactamase reporter (17). However, 2 μ M phenylarsine oxide, which contained an equivalent amount of arsenic but lacked the EDT antidote, was quite toxic. Therefore, EDT seems necessary and sufficient to prevent FLASH-EDT₂ from exerting acutely toxic effects (6, 7).

It is unlikely that every native protein that contains the core motif CCXXCC (8) will bind FLASH. The thiols must be able to reach an α -helical or other conformation able to form two pairs that grip the arsenics like pincers, yet the thiols must not be disulfide-bonded or tightly chelated to a metal. Endogenous competing proteins or ligands are clearly rare enough in mammalian cells to permit easy detection of transfected proteins over background (Figs. 3 and 4). FLASH confers temporal control of labeling, which is particularly helpful in quantifying FRET because it enables donor emission to be compared in situ before and after labeling by FLASH acting as an acceptor (Fig. 3). Derivatives of FLASH that incorporate luminescent or magnetic resonance reporters, environmentally sensitive fluorophores or indicators, or photochemically reactive moieties should label pro-

teins containing the CCXXCC motif and confer the appropriate spectroscopic properties. Immobilized FLASH analogs and tetracysteine motifs might complement systems such as biotin:avidin, glutathione:glutathione S-transferase, and nickel:polyhistidine for protein attachment and affinity purification (5, 18). FLASH dimers with two xanthene nuclei and four arsenics should induce homodimerization of peptides or proteins containing the tetracysteine motif in analogy to methods that rely on immunosuppressants binding to immunophilins (19). The new system combines high affinity and specificity, easy reversibility, easy modification of the ligand, small size of the peptide domain, physiological compatibility, and fluorescence indication that binding has occurred.

References and Notes

1. G. T. Hermanson, *Bioconjugate Techniques* (Academic Press, San Diego, CA, 1996).
2. R. Y. Tsien, *Annu. Rev. Biochem.* **67**, 509 (1998).
3. M. Chalfie and S. R. Kain, Eds., *GFP: Green Fluorescent Protein Strategies and Applications* (Wiley, New York, 1998).
4. J. L. Webb, *Enzyme and Metabolic Inhibitors* (Academic Press, New York, 1966) vol. III, pp. 595–819.
5. E. Kalef and C. Gitler, *Methods Enzymol.* **233**, 395 (1994).
6. V. P. Whittaker, *Biochem. J.* **41**, 56 (1947).
7. E. C. Webb and R. Van Heyningen, *ibid.*, p. 74.
8. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; E, Glu; R, Arg; W, Trp; and X, any amino acid.
9. G. Merutka, W. Shalongo, E. Stellwagen, *Biochemistry* **30**, 4245 (1991).
10. B. A. Griffin, thesis, University of California, San Diego (1998).
11. W. G. Lowe and C. S. Hamilton, *J. Am. Chem. Soc.* **57**, 1081 (1935).
12. C. Spang, F. T. Edlmann, M. Noltemeyer, H. W. Roesky, *Chem. Ber.* **122**, 1247 (1989).
13. R. Heim and R. Y. Tsien, *Curr. Biol.* **6**, 178 (1996).
14. A. Miyawaki, et al., *Nature* **388**, 882 (1997).
15. M. Ormö, et al., *Science* **273**, 1392 (1996).
16. F. Denizot and R. Lang, *J. Immunol. Methods* **89**, 271 (1986).
17. G. Zlokarnik, et al., *Science* **279**, 84 (1998).
18. C. F. Ford, I. Suominen, C. E. Glatz, *Protein Expr. Purif.* **2**, 95 (1991).
19. G. R. Crabtree and S. L. Schreiber, *Trends Biochem. Sci.* **21**, 418 (1996).
20. FLASH-EDT₂ was synthesized by reacting 4',5'-bis(ace-toxymercuri)fluorescein (fluorescein mercuric acetate) with 20 equivalents of arsenic trichloride (caution: highly toxic!) and 8 equivalents of di-isopropylethylamine in dry *N*-methylpyrrolidinone with palladium acetate as a catalyst. After 3 hours at room temperature, the reaction was quenched in pH 7 buffer with excess 1,2-ethanedithiol. The product was extracted into chloroform and chromatographed on silica gel with a 1:9 mixture of ethyl acetate and toluene. Trituration with ethanol gave an orange-red solid in 37% yield. ¹H nuclear magnetic resonance (at 200 MHz, in CDCl₃) peaks were as follows: δ 2.3 (s, OH), 3.57 (m, -SCH₂CH₂S-), 6.60 (d, H-2'), 6.69 (d, H-1'), 7.19 (d, H-7), 7.66 (m, H-5,6), 8.03 (d, H-4). Electrospray mass spectroscopy in negative ion mode indicated a monoisotopic mass for the -1 ion of 663.0 daltons (theoretical value, 662.85). The extinction coefficient was 4.1×10^4 M⁻¹ cm⁻¹ at 507.5 nm at pH 7. The peptide Ac-Trp-Glu-Ala-Ala-Ala-Arg-Glu-Ala-Cys-Cys-Arg-Glu-Cys-Cys-Ala-Arg-Ala-amide was prepared by standard solid-phase methods and purified by *n*-butanol/water countercurrent chromatography.

REPORTS

21. ECFP (14) is *Aequorea victoria* GFP with mammalian codons and the following additional mutations: K26R, F64L, S65T, Y66W, N146I, M153T, V163A, and N164H (8). A gene encoding a fusion of the peptide Ala-Glu-Ala-Ala-Ala-Arg-Glu-Ala-Cys-Cys-Arg-Glu-Cys-Cys-Ala-Arg-Ala to the COOH-terminus of ECFP was created with the following primer in a polymerase chain reaction (PCR): 5'-GCCGAATCTTAGGCCTGGCGCAGCACTCCCTGCAGCAGGCCTCCCTCGCGCGGCTCGGCCTGTACAGCTCGTCCATGCCG-3'. The resulting gene was inserted into the

pcDNA3 vector (Invitrogen) at Hind III and Eco RI restriction sites. After amplification in DH5 bacteria, it was transfected into HeLa cells with Lipofectin (Gibco-BRL). The gene for *Xenopus* calmodulin was mutated to encode cysteines at residues 6, 7, 10, and 11 by PCR with the following primer: 5'-CGCGGATC-CGCCACCATGCATGACCAACTGACATGCTGCCAGATTTCGCTCTCAAGAGAGCTTCTCATATTTC-3', and inserted into pcDNA3 as described above.

22. Images of cells at room temperature were acquired with a cooled charge-coupled device camera (Photo-

metrics, Tucson, AZ) controlled by Metafluor software (Universal Imaging, West Chester, PA).

23. Supported by NIH (grant NS27177 to R.Y.T. and a traineeship to B.A.G. from grant T32 CA09523) and the Howard Hughes Medical Institute. R.Y.T. is a consultant for Aurora Biosciences Corp. We thank L. A. Gross for mass spectra, A. Miyawaki for help with molecular biology, and T. Knapp for help with toxicity testing.

11 March 1998; accepted 8 June 1998

Rad53 FHA Domain Associated with Phosphorylated Rad9 in the DNA Damage Checkpoint

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The Rad53 protein kinase of *Saccharomyces cerevisiae* is required for checkpoints that prevent cell division in cells with damaged or incompletely replicated DNA. The Rad9 protein was phosphorylated in response to DNA damage, and phosphorylated Rad9 interacted with the COOH-terminal forkhead homology-associated (FHA) domain of Rad53. Inactivation of this domain abolished DNA damage-dependent Rad53 phosphorylation, G₂/M cell cycle phase arrest, and increase of *RNR3* transcription but did not affect replication inhibition-dependent Rad53 phosphorylation. Thus, Rad53 integrates DNA damage signals by coupling with phosphorylated Rad9. The hitherto uncharacterized FHA domain appears to be a modular protein-binding domain.

The consequences of DNA damage to eukaryotic cells are minimized by simultaneous activation of DNA repair mechanisms and cell cycle arrest at DNA damage checkpoints. In *Saccharomyces cerevisiae*, the proteins encoded by the genes *POL2*, *RFC5*, and *DPB11* are required for arrest in response to inhibition of DNA replication (1), whereas the proteins encoded by the genes *RAD9*, *RAD17*, *RAD24*, *DDC1*, and *MEC3* operate in response to DNA damage (2, 3). *MEC1* and *RAD53* are required for both checkpoints (3, 4). Rad53 (also called Spk1, Sad1, and Mec2) is an essential Ser/Thr/Tyr protein kinase (5, 6). Mec1 belongs to a subgroup of the phosphatidylinositol-(3)-phosphate kinase family that includes known protein kinases (7). Signals generated by replication inhibition or by DNA damage apparently activate a protein kinase cascade, in which Mec1 is required for phosphorylation of Rad53 (8, 9), which is required for phosphorylation and activation of protein kinase Dun1 (10).

A catalytically hypoactive Rad53, in which Ala²⁰⁸ was changed to Pro (11), was

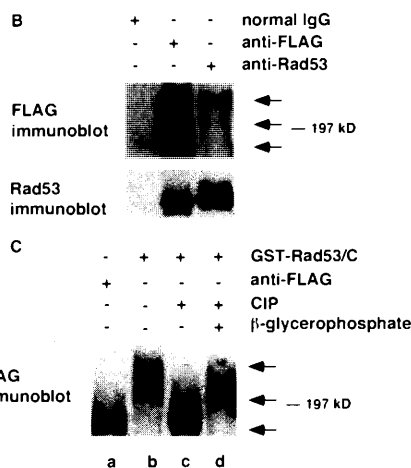
used to search for interacting proteins expressed from a *S. cerevisiae* cDNA library (S. J. Elledge, Baylor College of Medicine) in a yeast two-hybrid screen. The strongest interactor was a region of Rad9 (amino acids 553 to 1056) followed by polyadenylate. Au-

thentic Rad9 and Rad53 also interacted with one another (Fig. 1A).

Rad9-FLAG (12) overexpressed with Rad53 migrated on gels as a heterogeneous polypeptide. The fastest migrating form (estimated molecular size just below 200 kD) predominated (Fig. 1, B and C) and was larger than the 149-kD size of the predicted sequence (13). These bands were only present in galactose-treated samples from strains carrying the tagged expression plasmid. The fastest migrating form of Rad9 migrated at a similar position to that of the in vitro transcription-translation product (14). Antibody to FLAG (anti-FLAG) cross-precipitated Rad53. Anti-Rad53 preferentially precipitated slower migrating forms of Rad9 (Fig. 1B). Bacterially produced glutathione S-transferase (GST)-Rad53 COOH-terminus also interacted with slow migrating forms of Rad9 from yeast lysates overexpressing both Rad9 and Rad53 (Fig. 1C). Calf intestine alkaline phosphatase (CIP) converted the slow migrating forms to the fast migrating form, indicating that the mobility shift of Rad9 is mostly

BD	Rad9		Rad53		-	
	Rad9	Rad53	Rad9	Rad53	Rad9	Rad53
AD	-	Rad53	-	Rad9	Rad53	Rad9
	1	299	1	221	5	2

Fig. 1. Interaction of Rad9 and Rad53. (A) Two-hybrid analysis. β -Galactosidase (β -Gal) activity resulting from coexpression of *GAL4* DNA-binding domain (BD) and *GAL4* activation domain (AD) fusion constructs (pPC86 and pPC97) (22) of *RAD9*-FLAG (encoding Rad9 followed by a single epitope tag) and *RAD53* was assayed in strain MaV103 (*GAL1::HIS3*, *GAL1::LacZ*, *SPAL::URA3*) (22) as described (23). Arbitrary units are defined as before (23). -, empty vector control. β -Gal activity is shown in $\times 10^{-3}$ units. **(B)** Coimmunoprecipitation of Rad9-FLAG and Rad53. pRS313*GAL1*-*RAD9*-FLAG and pNB187-*RAD53* (6) were transformed into YMP10500 (*MATa ura3 leu2 trp1 his3*, provided by E. Foss and L. Hartwell, Fred Hutchinson Cancer Research Center). Expression of Rad9-FLAG and Rad53 was induced by incubating log phase cells in galactose (2%) for 3 hours. Equal portions of extracts from 100-ml cultures were immunoprecipitated and immunoblotted by means of detection with enhanced chemiluminescence. Arrows mark positions of forms of Rad9-FLAG precipitated with anti-Rad53. Bar marks position of a 197-kD marker. **(C)** Rad9 is phosphorylated. GST-Rad53/450-821 fusions were produced in *Escherichia coli* and bound to glutathione-Sepharose beads. Equivalent portions of yeast lysates prepared as in (B) were used for GST-affinity purification. One-fifth equivalent amount of lysate was precipitated with anti-FLAG. Beads were incubated with 50 units of CIP at 37°C for 10 min in the presence or absence of β -glycerophosphate, a CIP inhibitor (24). Samples were then analyzed by immunoblotting. Arrows mark forms of Rad9-FLAG. Bar indicates the position of a 197-kD marker.



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