

hammerhead ribozyme nor to these metal ions. Any RNA for which an assay is available to monitor metal exchange can, in principle, be used. Folded RNAs provide complex surfaces to which different metal ions can bind, with each site having its own specificity. The binding site observed with one competitor, therefore, may not necessarily be the same as that observed with another. Because few RNAs have been crystallographically characterized, the current challenge is to develop structural or functional methods to identify unique metal ion sites on these complex folded RNA structures.

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- Saturating concentrations of ribozyme (500 nM) and trace ³²P-labeled substrate were annealed for 90 s at 90°C in 50 mM Pipes (pH 7.5), and then allowed to cool to 25°C. Reactions were initiated by adding MgCl₂ to the appropriate final concentration. When Tb(III) was present, it was added concomitantly with the Mg(II). Reactions were quenched by diluting a 10-μl portion of the reaction mixture into 20 μl of stop buffer (100 mM EDTA, 7 M urea, 1 × tri-borate-EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol, 0.1% orange G). Reaction products were analyzed by electrophoresis on denaturing 20% polyacrylamide gels and quantitated with a Molecular Dynamics Phosphorimager and ImageQuant software. Pseudo-first order rate constants were calculated with Kaleidagraph (Synergy Software) from nonlinear least-squares fitting of plots of the fraction product [product/(product + substrate)] versus time. For extremely slow reactions, initial rates derived from linear fits of the same plots were used to estimate the rate constants. Protracted reactions (>4 to 6 hours) in the presence of Tb(III) were problematic because of nonspecific degradation of the RNA under the conditions used for kinetic analysis (22). Standard 1-hour time courses showed no substantial degradation. Reactions were usually performed in triplicate and the data averaged to give the observed rate constant. Errors are reported as standard deviations of the mean from replicate experiments.
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Quantitation of Transcription and Clonal Selection of Single Living Cells with β-Lactamase as Reporter

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Gene expression was visualized in single living mammalian cells with β-lactamase as a reporter that hydrolyzes a substrate loaded intracellularly as a membrane-permeant ester. Each enzyme molecule changed the fluorescence of many substrate molecules from green to blue by disrupting resonance energy transfer. This wavelength shift was detectable by eye or color film in individual cells containing less than 100 β-lactamase molecules. The robust change in emission ratio reveals quantitative heterogeneity in real-time gene expression, enables clonal selection by flow cytometry, and forms a basis for high-throughput screening of pharmaceutical candidate drugs in living mammalian cells.

Biological specificity is mediated by the precise and selective regulation of gene expression in response to intrinsic developmental programs and extrinsic signals. To

understand the regulation of gene expression, it is essential to use an assay of high sensitivity and fidelity that reports expression at the level of the single living cell.

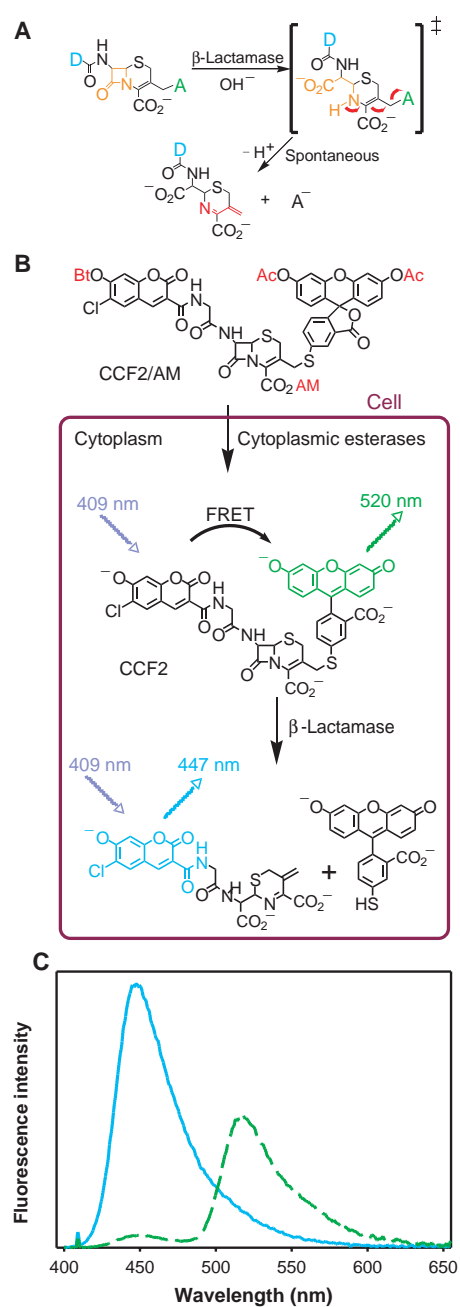
Such resolution is beneficial even in nominally homogeneous or clonal cell populations, because individual cells often prove quite heterogeneous in phenotype. A non-destructive assay compatible with high-throughput methods such as fluorescence-activated cell sorting (FACS) would enable powerful genetic screens for isolating individual variants with defined transcription responses. Correlation of gene expression with physiological responses and developmental fates would be facilitated by transcriptional assays that do not jeopardize cell survival. Current assays based on the reporter genes chloramphenicol acetyltransferase, secreted alkaline phosphatase, β -galactosidase, and firefly luciferase (1) typically require cell permeabilization or lack single-cell resolution, whereas the green fluorescent protein (GFP) is a relatively insensitive reporter due to lack of enzymatic amplification (2).

Here we demonstrate the use of β -lactamase and a membrane-permeant, fluorogenic substrate ester to measure gene expression in single live mammalian cells with high sensitivity and real-time response. β -Lactamases are a family of bacterial enzymes that cleave penicillins and cephalosporins very efficiently, in some cases at rates that approach the ultimate diffusion-controlled limit (3). Because of their clinical importance in mediating antibiotic resistance, much is known about their three-dimensional structure, mechanism, optimal substrates, and inhibitors (4, 5). A well-characterized isoform is the 29-kD, plasmid-encoded TEM-1 β -lactamase from *Escherichia coli*, the product of the ampicillin resistance gene *Amp^r* (6). This enzyme normally is secreted into the periplasmic space but accumulates in bacterial cytoplasm when its signal sequence is genetically deleted (7). Although colorimetric substrates for β -lactamase are known (8–11), fluorogenic substrates that can detect the enzyme within living cells have not been reported. We wanted a substrate that would report enzymatic cleavage with a large shift in fluorescence emission wavelength, not just an overall change in brightness. Ratios of intensities at two or more wavelengths would then indicate the extent of enzyme-mediated cleavage with minimal interference from variations in cell size, probe con-

Fig. 1. β -Lactamase-catalyzed hydrolysis of a cephalosporin monitored by a change in fluorescence emission of a substrate. **(A)** β -Lactamase catalyzes the hydrolysis of a cephalosporin β -lactam ring (orange), generating an unstable intermediate (in brackets). The intermediate spontaneously rearranges to products in which donor (D) and acceptor (A) are separate. **(B)** Schematic representation of substrate loading into cells in culture. CCF2/AM diffuses across the cell's plasma membrane where cytoplasmic activities catalyze the hydrolysis of its ester functionalities (Ac, acetyl; Bt, butyryl; AM, acetoxymethyl), releasing the polyanionic β -lactamase substrate CCF2, which is trapped due to its charges. In the intact substrate, excitation of the coumarin donor at 409 nm leads to fluorescence resonance energy transfer (FRET) to the fluorescein acceptor and reemission of green light. β -Lactamase-catalyzed hydrolysis of the substrate separates donor and acceptor. The donor then emits blue fluorescence whereas the acceptor is quenched. **(C)** Corrected emission spectra of equal concentrations of CCF2 (dashed green line) and its hydrolysis product (solid blue line) in PBS at pH 7.4 with excitation at 409 nm.

centration, excitation intensity, and emission sensitivity.

Cleavage of the β -lactam ring of a cephalosporin creates a free amino group, which triggers spontaneous elimination of any leaving group previously attached to the 3' position (4) (Fig. 1A). We reasoned that if two fluorophores with suitably overlapping spectra were attached to the 7 and 3' positions of a cephalosporin, they would be held close together and would exhibit efficient fluorescence resonance energy transfer (FRET) (12). Excitation of the shorter wavelength fluorophore, the donor D, would result in emission from the longer wavelength fluorophore, the acceptor A. However, β -lactamase attack would split off the fluorophore on the 3' position, disrupt FRET, and reestablish fluorescence emission from the donor. We designed and synthesized (13) a β -lactamase substrate called CCF2 and a membrane-permeant ester derivative CCF2/AM (Fig. 1B) with the desired properties. The donor fluorophore in CCF2 is 7-hydroxycoumarin, with a 6-chloro substituent to lower the acid constant (pK_a) to 5.1 and make the fluorescence independent of pH values above 6. A glycine was inserted between the coumarin and the cephalosporin so that the fluorophore did not obstruct β -lactamase attack. The coumarin was attached to the 7 position rather than the 3' position of the cephalosporin, so that the polar groups of the latter help retain the donor inside live cells even after β -lactamase attack. The FRET acceptor, fluorescein, was attached through a stable thioether linkage, which minimizes the rate of spontaneous aqueous hydrolysis. An additional benefit of the thioether is



that when it is cleaved, the resulting free thiol group almost completely quenches the fluorescence of the fluorescein (14). Figure 1C shows the fluorescence emission spectra of CCF2 before and after cleavage by β -lactamase. The 7-hydroxycoumarin donor is an efficient light antenna with a molar extinction coefficient of 40,000 $\text{dm}^3/\text{mol}\cdot\text{cm}$ at its 409-nm peak. In the intact substrate, the donor is 95% quenched and the fluorescein acceptor reemits as green light 32% of the photons that excited the donor. After β -lactamase attack has released the fluorescein, the coumarin attached to the cephalosporin remnants glows bright blue with a fluorescence quantum yield of 0.74, whereas the mercaptofluores-

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cein has negligible fluorescence because it hardly absorbs 409-nm light and is further quenched by its free thiol. The ratio of the emission intensity at 447 nm (blue) to that at 520 nm (green) is a concentration-independent measure of the extent of the reaction and increases 70-fold upon complete conversion. In phosphate-buffered saline (PBS) at pH 7.4, CCF2 is hydrolyzed by TEM-1 β -lactamase with catalytic constant (k_{cat}) = $29 \pm 1 \text{ s}^{-1}$ and Michaelis constant (K_m) = $23 \pm 1 \mu\text{M}$ (Fig. 2A; values ob-

tained from weighted least squares fit), compared with a spontaneous hydrolysis rate of $1.2 \cdot 10^{-7} \text{ s}^{-1}$. Because of the $>10^8$ -fold enzymatic acceleration, β -lactamase concentrations down to 5 fM are readily quantifiable with CCF2 in vitro (Fig. 2B).

CCF2/AM is a fully esterified derivative of CCF2 that is colorless, nonfluorescent, and nonpolar enough to cross cell membranes. In the cytosol, its four ester groups hydrolyze, probably through catalysis by nonspecific esterases, releasing and trapping

CCF2, which is the actual fluorescent substrate for β -lactamase. When Jurkat cells were incubated in aqueous buffer with $1 \mu\text{M}$ CCF2/AM for 1 hour at room temperature, CCF2 accumulated to 50 to $100 \mu\text{M}$ cytoplasmic concentration. CCF2 fluorescence in these cells was about 85 times that of cellular autofluorescence. Other cell lines loaded under these conditions were (listed with fluorescence relative to endogenous background) HeLa (120 \times), HEK (human embryonic kidney) 293 (75 \times), GH3 (56 \times), COS-7 (44 \times), CHO (chinese hamster ovary) (38 \times), and CV-1 (28 \times). Jurkat cells at room temperature retained 50% of the initial CCF2 after 2.5 hours and 25% after 10 hours, whereas the blue cleavage product was 50% retained at 1.5 hours and 25% at 7 hours. In CHO cells, the blue product was retained better than the green substrate. Probenecid, a standard inhibitor of nonspecific anion transport (15), greatly improved retention of both substrate and product. Both rapidly leaked out of lysed cells, so that damaged cells were nonfluorescent and were readily distinguished from live cells not expressing the reporter.

TEM-1 β -lactamase was made a cytoplasmic resident protein by replacement of the bacterial signal presequence by Met-Ser or Met-Gly (7, 11). Mammalian cells were transfected with this reporter gene together with transcription-regulating sequences to show how different amounts of β -lactamase expression correlated with ability to convert green CCF2 fluorescence into blue (Fig. 3). For example, Jurkat lymphocyte controls lacking transfected β -lactamase remained green-fluorescent even over 16 hours (Fig. 3A). A clone (C84) of Jurkat cells containing the β -lactamase reporter under the control of a tandem trimer of nuclear factor of activated T cells (NF-AT) binding sites (16, 17) expressed an average of 50 β -lactamase molecules per cell as measured enzymatically in lysates. This very low amount of expression, resulting from leakiness of the NF-AT promoter, caused the CCF2-loaded cells to turn blue-green in 16 hours (Fig. 3B). Another clone (C2) of Jurkat cells was constructed with the same NF-AT- β -lactamase reporter and the M_1 muscarinic receptor gene under the control of a constitutive promoter. Unstimulated C2 cells had 150 to 300 copies of β -lactamase per cell, presumably because the M_1 receptors slightly activated the Ca^{2+} and protein kinase C signaling pathways to which NF-AT is responsive. This amount of expression sufficed to generate blue fluorescence in most of the cells over 16 hours, although at 1 hour they were still mostly green (compare Fig. 3, C and D). When the muscarinic agonist carbachol was applied, the blue fluorescence was apparent in most

Fig. 2. Hydrolysis of CCF2 by TEM-1 β -lactamase. (A) Double-reciprocal plot of CCF2 molecules hydrolyzed per enzyme molecule per second (v) versus substrate concentration ($[\text{CCF2}]$). The rate of increase in blue fluorescence (460 nm, bandwidth 50 nm) with violet excitation light (395 nm, bandwidth 20 nm) was used as a measure of the rate of CCF2 hydrolysis. Seven substrate concentrations bracketing the K_m were prepared in PBS. Kinetics measurements were taken on a Cytofluor 4000 microtiter plate fluorimeter (Perseptive Biosystems) in quadruplicate with less than 5% substrate converted. The data were corrected for optical inner filtering of the solutions above substrate concentrations of $10 \mu\text{M}$. Error bars indicate standard errors. (B) Linearity of the assay with enzyme concentration. Twofold serial dilutions of TEM-1 β -lactamase in PBS were prepared in a 96-well clear-bottom plate. The rate of hydrolysis of CCF2 in $100 \mu\text{l}$ of a $10 \mu\text{M}$ solution was monitored by fluorescence as above in triplicate. Solid circles represent data obtained from 5-min readings, open circles from 5-hour readings. Bars indicate standard errors.

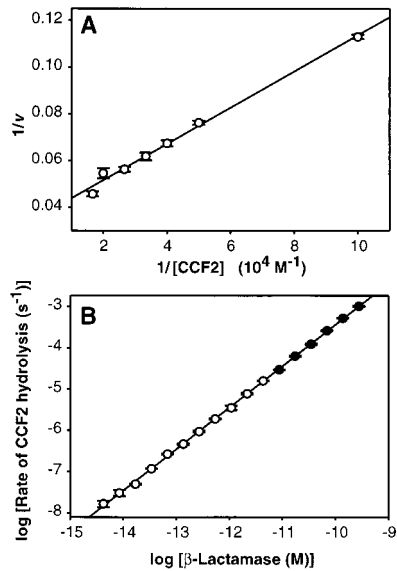
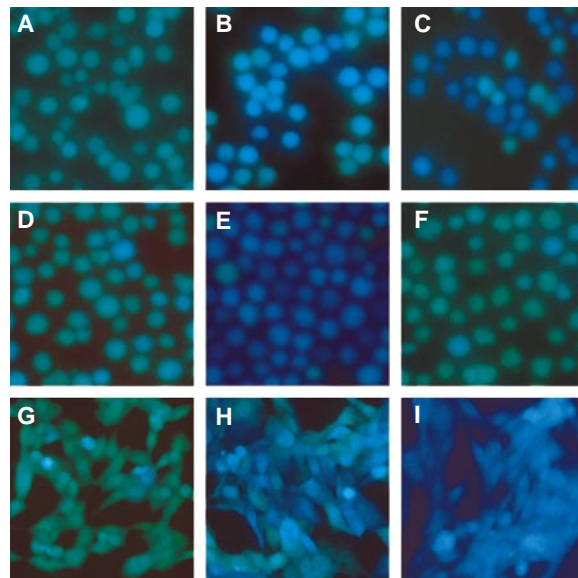


Fig. 3. Receptor-regulated gene expression in living mammalian cells visualized by β -lactamase-catalyzed hydrolysis of CCF2. (A to C) Cells were loaded with $1 \mu\text{M}$ CCF2/AM in RPMI 1640 with 10 mM HEPES, transferred to fresh medium containing 2.5 mM probenecid, and kept at room temperature for 16 hours. (A) Wild-type Jurkat cells. (B) Jurkat cell clone C84 stably transfected with the TEM-1 β -lactamase gene regulated by a promoter containing a trimer of NF-AT-binding sites (NF-AT-bla). (C) Jurkat clone C2 stably transfected with NF-AT-bla and the M_1 muscarinic receptor under the control of a cytomegalovirus promoter. (D to F) Cells were loaded with $1 \mu\text{M}$ CCF2/AM in Hanks' balanced salt solution (HBSS) for 1 hour at 22°C and viewed immediately afterward. (D) Jurkat clone C2. (E) Clone C2 pretreated with $100 \mu\text{M}$ carbachol for 4 hours at 37°C . (F) Same as (E) but with $10 \mu\text{M}$ pirenzepine in addition to carbachol. (G to I) BHK cells, stably expressing TEM-1 β -lactamase under the control of the mouse mammary tumor virus promoter (MMTV-bla), grown in Dulbecco's modified Eagle's medium with 10% steroid-depleted fetal bovine serum (FBS), then loaded with $10 \mu\text{M}$ CCF2/AM in HBSS at 22°C for 1 hour and viewed 1 hour later. (G) No stimulation. (H) Stimulation for 16 hours at 37°C with $1 \mu\text{M}$ dexamethasone in medium containing 5% complete FBS just before loading. (I) A blue subclone grown from the cells in (H), retested under the same stimulation conditions. (A) to (F) are $100 \mu\text{m}$ wide, (G) to (I) are $200 \mu\text{m}$ wide.



of the C2 cells within 1 hour of CCF2/AM loading (Fig. 3E). Such rapid conversion corresponded to 15,000 to 20,000 β -lactamase molecules per cell. Thus, the sensitivity of the in situ assay could be adjusted simply by varying the time allowed for β -lactamase action. The muscarinic antagonist pirenzepine prevented stimulation by carbachol (Fig. 3F). Adherent cells and nuclear receptors were equally amenable to this assay. For example, BHK (baby hamster kidney) cells, transfected with the reporter gene under the control of a promoter containing a pentamer of glucocorticoid response elements from mouse mammary tumor virus, remained almost entirely green without hormone (Fig. 3G). Dexamethasone stimulation caused most cells to turn blue (Fig. 3H) without requiring transfection of exogenous glucocorticoid receptors (18). Because the cells were not injured by the dye loading, it was a simple matter to use a suction pipette to pick blue-fluorescing cells and subclone them. A population that was essentially 100% responsive to dexamethasone was obtained (Fig. 3I).

For systematic engineering of cell lines with desired patterns of gene expression, flow cytometry of the blue/green ratio in viable cells offers an automated selection procedure to complement or replace traditional selection based on antibiotic resistance (19). For example, Jurkat cells containing the NF-AT- β -lactamase reporter, similar to C84, were transfected with the M_1 receptor and a G418 resistance gene in the same pcDNA3 vector. After 3 weeks of selection in G418 medium, only about 40% of the cells were responsive to carbachol. Isolation of one of the blue cells by FACS then produced the C2 subclone (Fig. 3, C to F), in which ~80% of cells responded to carbachol, a percentage stable for more than 50 passages without antibiotics. Sorting can isolate even rarer blue or green cells. When β -lactamase-expressing cells were artificially mixed with up to a 10^6 -fold excess of nonexpressing cells, a FACS was readily able to detect 50% and recover 30% of the positive cells for further culturing. Stained cells of either color were no different from unstained cells in subsequent viability and proliferation (20).

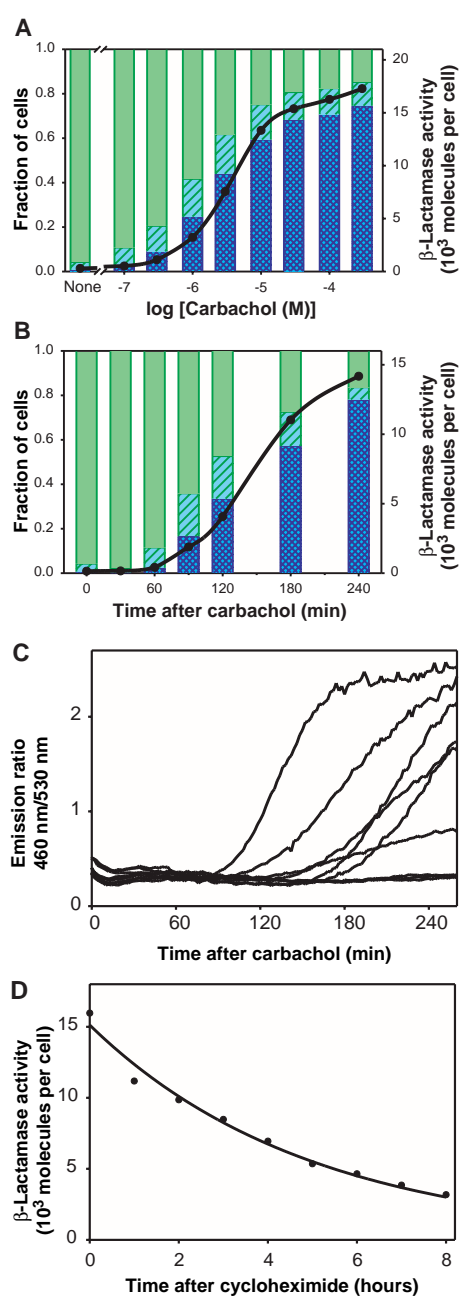
The β -lactamase assay also revealed the dose-response and kinetic behavior of gene transcription at both the population and single-cell levels. We compared the average β -lactamase content of C2 Jurkat cells (measured in cell lysates) with the percentages of blue, blue-green, and green cells measured by flow cytometry as a function of carbachol dose and as a function of time after stimulation with a maximal dose of carbachol (Fig. 4, A and B). The percentage of individual cells with intermediate (blue-

green) colors remained fairly low even at early times and lower doses when the lysates showed only partial activation in the population. Thus, the increase in total amount of gene expression occurred mainly through recruitment to the fraction of blue cells expressing large amounts rather than a synchronized gradual increase in reporter content in all the cells. We could also watch the stimulus-evoked color change develop progressively in individual cells (Fig. 4C). Although all the cells had been subcloned from one highly responsive ancestor isolated by FACS, they showed different latencies before abruptly increasing their ratio of blue to green emissions. These results are consistent with proposals that gene expres-

sion occurs in abrupt stochastic bursts (16, 21–23).

We determined the lifetime of intracellular β -lactamase, which is important for its ability to track reductions in expression, by expressing the enzyme under the control of a constitutive viral promoter and measuring how rapidly the enzyme activity decayed after interruption of protein synthesis. The present β -lactamase decayed with a half-life of 206 ± 12 min (Fig. 4D), similar to that of firefly luciferase (24), which is often used for reporting transient gene expression. Known β -lactamases include forms with both higher and lower thermostability (25) than TEM-1 used here, so it should be possible to find isozymes with longer or

Fig. 4. (A) Agonist dose-response in Jurkat clone C2 stably transfected with M_1 muscarinic receptor and NF-AT- β -lactamase reporter genes. After 4 hours of incubation with the indicated concentrations of carbachol at 37°C, cells were collected, loaded with 1 μ M CCF2/AM for 1 hour at room temperature, and analyzed by FACS [Becton-Dickinson FACS Vantage; excitation 351 to 364 nm, emission 460/50 nm (blue) and 535/40 nm (green)]. The ratio of uncompensated intensities was used to classify cells (corrected by a factor of 0.2 to allow comparison to imaging data in Fig. 4C). Cross-hatched bars indicate blue cells, high β -lactamase activity, ratios 1.8 to 4.0. Hatched bars indicate blue-green cells, medium activity, ratios 0.8 to 1.8. Solid green bars represent green cells with little to no β -lactamase activity, ratios of 0.1 to 0.8. Matching samples of cells were lysed by three cycles of freeze-thawing in PBS. β -Lactamase activity in the lysate was measured from the rate of hydrolysis of 1 nmol of CCF2 in 100 μ l in a Cytofluor 4000 microtiter plate fluorimeter (PerSeptive Biosystems) (●). (B) Time course of agonist response in clone C2. Cells were treated with 100 μ M carbachol at 37°C. At the indicated times cells were harvested and analyzed as in (A). (C) Real-time imaging of gene expression in single live cells. C2 Jurkat cells were exposed to 100 μ M carbachol at 35°C in the continuous presence of 1 μ M CCF2/AM in the medium (RPMI 1640 containing phenol red and 10 mM Hepes) on the stage of a fluorescence microscope digital imaging system. Individual cells were imaged through a 20 \times objective and their fluorescence emission ratio recorded over time after carbachol addition. (D) Stability of β -lactamase in Jurkat cells. Cells of a Jurkat clone stably expressing β -lactamase with a cytomegalovirus promoter were treated with cycloheximide (40 μ g/ml) to stop new synthesis of the enzyme. Cells were kept at 37°C and sampled hourly. β -Lactamase activity in lysates was determined as in (A).



shorter half-lives.

Transcriptional readouts are versatile screens for drug candidates, because most signaling pathways result in expression or repression of specific response elements and genes. Steps in disease progression likewise involve modulation of expression patterns, which can be monitored by transcriptional reporters even if the disease-causing mechanisms are not yet understood. The selectivity and sensitivity of the β -lactamase transcriptional readout as a screen for drugs was assessed with a library of known pharmacophores (Microsource Discovery Systems, Gaylordsville, Connecticut). This library of 480 biologically active compounds was tested in a blind screen for muscarinic agonism and antagonism in 96-well microplates containing the C2 cell line. Known agonists (acetylcholine, arecholine, bethanechol, carbachol, methacholine, and pilocarpine) were identified at a test concentration of 5 μ M, and no other compounds in this set displayed agonist activity. In the primary screen for antagonist activity, 41 compounds were active (>80% inhibition of the response induced by 100 μ M carbachol). Rescreening in a cell line lacking the M_1 receptor distinguished the 13 true receptor antagonists from 28 compounds that inhibited signal transduction further downstream.

A concentration of about 50 β -lactamase molecules per cell, or 60 pM in a 1.4-pl Jurkat cell, produces in 16 hours a blue-green ratio well above that of non-transfected cells, as apparent by unaided color vision or color film (Fig. 3). By decreasing the duration of exposure to substrate, we measured expression levels up to about 20,000 molecules per cell in situ. A yet higher dynamic range was quantifiable in lysates (Fig. 2B). GFP is the only reporter of single-cell gene expression whose assay is less invasive than that of β -lactamase, but because GFP is not catalytic, about 1 μ M cytosolic GFP (10^5 to 10^6 molecules per cell) is necessary to show up over autofluorescence background (26). Thus, most applications of GFP in mammalian cells have needed strong constitutive promoters, typically from viruses, rather than weaker promoters responsive to native mammalian signal transduction pathways. However, β -lactamase does not yet supplant GFP as a tag for subcellular localization of cytoplasmic fusion proteins, because CCF2 and its cleavage product diffuse throughout the cytosol. The β -lactamase reporter system will facilitate many applications such as genetically tagging transfected mammalian cells, engineering cell lines with specific expression phenotypes, expression cloning of molecules that can be linked to gene transcription, trapping genes whose expression levels

are developmentally or pharmacologically regulated, and identifying natural or pharmaceutical ligands for receptors.

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Dimerization-Induced Inhibition of Receptor Protein Tyrosine Phosphatase Function Through an Inhibitory Wedge

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The function and regulation of the receptorlike transmembrane protein tyrosine phosphatases (RPTPs) are not well understood. Ligand-induced dimerization inhibited the function of the epidermal growth factor receptor (EGFR)-RPTP CD45 chimera (EGFR-CD45) in T cell signal transduction. Properties of mutated EGFR-CD45 chimeras supported a general model for the regulation of RPTPs, derived from the crystal structure of the RPTP α membrane-proximal phosphatase domain. The phosphatase domain apparently forms a symmetrical dimer in which the catalytic site of one molecule is blocked by specific contacts with a wedge from the other.

The RPTPs are a family of signaling molecules whose function and regulation are not well understood (1). In T cells, the RPTP CD45 is required for T cell development (2) and T cell receptor (TCR) signal transduction (3-5), presumably by dephosphorylating the negative regulatory COOH-terminal tyrosine in the Src-family kinase Lck (6). A chimeric EGFR-CD45 molecule restores TCR-mediated signal transduction in a CD45-deficient T cell line; furthermore, treatment of these cells with EGF blocks TCR-mediated signaling, which suggests that CD45 is negatively

regulated by ligand-induced dimerization (7). A possible explanation for this negative regulation comes from the crystal structure of the membrane-proximal phosphatase domain of the RPTP, RPTP α , which revealed a putative inhibitory wedge in symmetrical dimers (8). Two acidic residues found in this wedge are strongly conserved among the membrane-proximal phosphatase domains of RPTPs (8). Thus, ligand induced-dimerization may result in inhibition of phosphatase activity, and consequently of signaling function, through specific interactions between