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13. ABSTRACT *(Maximum 200 Words)*

The focus of this project is to develop a system for screening for novel drugs and drug targets to inhibit the progression of breast cancer. Our approach capitalizes on recent findings in our laboratory that should allow new types of screens for drugs. These screens should enable the identification of drugs that arrest the growth of breast cancer cells as well as the identification of new drug targets. Specifically, certain growth factor receptors will be targeted that are known to be present in abundance on the surface of breast cancer cells. These receptors have been designated as the ErbB family of receptors. Cancers expressing an abundance of one of these receptors, the ErbB2 receptor, have been shown to be resistant to conventional therapy. Very recently a drug that targets ErbB2 directly has been found to increase patient survival in a phase III clinical trial. The identification of additional drugs or small molecules that target this family of receptors and impair their function should greatly enhance our ability to successfully treat patients with this form of cancer.

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Introduction

Extensive clinical and biochemical evidence has implicated the transmembrane tyrosine kinase ErbB-2 receptor in the pathogenesis of multiple common carcinomas. In particular, analyses of the prognostic value of ErbB-2 in breast cancer revealed an inverse correlation between ErbB-2 expression and responsiveness to conventional therapy. Furthermore, recent evidence suggests that therapies directly targeting ErbB-2 significantly increase patient survival. ErbB-2 functions as a co-receptor for other ErbB family members. ErbB-2 appears to take part in an extensive network of heterodimeric interactions, resulting in the enhancement of the signals triggered by a multi-member family of growth factors (EGF-like factors). The general aim of this research project is to develop a novel test system to screen for drugs that will antagonize ErbB receptor function in breast cancer. Specifically a method developed in our laboratory for monitoring protein interactions in live cells *in situ* is being applied to this family of receptors in cancer cells. The approach capitalizes on "reporter subunit complementation" using beta-galactosidase. Receptor dimerization, the first step in the signaling pathway, is being monitored in live cells over time. This system should allow screens for drugs (antagonists) that target specific ErbB heterodimers with therapeutic applications to breast cancer.

Body/Key Research Accomplishments

Monitoring Dimerization of the EGF Family of Receptors Involved in Breast Cancer

During the first year of this grant, we have made substantive progress toward our goals. As a first step, we have focused primarily on the construction of chimeric proteins comprising truncated versions of the members of the ErbB family of tyrosine kinase receptors and the $\Delta\omega$ and $\Delta\alpha$ β-galactosidase (β-gal) mutants. We were successful in constructing $\Delta\omega$ and $\Delta\alpha$ fusions with three of the four ErbB receptors, specifically: ErbB1 (EGFR), ErbB2 and ErbB3. We are in the process of acquiring the cDNA for ErbB4 by a reverse-transcription PCR approach which has proved less efficient than predicted, possibly due to the length of the cDNA that needs to be amplified.

Retroviral vectors have been constructed containing the chimeric protein sequences and these sequences have been tested by infecting the C2C12 myoblast cell line. Expression of the chimeric proteins in these cells has been confirmed by Western blot with both antibodies to the relevant ErbB family member and to β-gal. The chimeric proteins were then expressed pairwise in C2C12 cells, in order to of monitor both their homodimerization and heterodimerization.

To date, the analysis of the homodimerization of ErbB1 has been completed and published (Blakely et al., 2000). We found that treatment of the cells with EGF-like compounds for as little as one minute resulted in chimeric receptor dimerization detectable as an increase in enzymatic β-gal activity. The dose response of appearance of β-gal activity in response to different ErbB1 ligands was consistent with the published affinities of the ligands for the receptor, suggesting that the β-gal moiety of the chimaric proteins does not affect their dimerization significantly. β-gal complementation was reversible upon removal of ligand and could be reinduced, demonstrating that the kinetics of dimerization can be monitored. Antibodies that block ligand binding inhibited receptor dimerization and blocked β-gal complementation, suggesting that this system can be used to identify inhibitors of ErbB receptor activation. These results demonstrate that β-gal complementation provides a rapid, simple, and

sensitive assay for protein interactions at the membrane and validates our approach to the other members of the ErbB family.

We have now focused on monitoring the heterodimerization of the ErbB2-ErbB1 and ErbB2-ErbB3, as these pairs of receptors are likely to be the most relevant to breast cancer. Cell lines expressing both proteins have been established and encouraging preliminary results have been obtained using polyclonal populations. We are now in the process of isolating subclones that will allow us to monitor these dimerization events in detail upon treatment with a range of ligands.

Construction of a Mammalian Two-Hybrid System in Order to Identify Novel Targets for Breast Cancer

Another objective of the proposed research is to create a method for screening for novel proteins which interact with the EGF family of receptors. The screen we are devising consists of the EGF receptor fused to one of the β-gal mutants and a cDNA library fused to the complementing β-gal mutant. Interaction of a protein with the EGF receptor will result in an increase in β-galactosidase activity which can be detected using the fluorescence activated cell sorter and a fluorescent substrate of β-gal. Cells with high β-gal activity will be isolated and their library cDNA analyzed. This method permits screening in mammalian cells, and allows the protein of interest to be studied in its natural context, at the plasma membrane.

We have investigated several properties of the β-gal system for their compatibility with the aforementioned screen. Given the method of complementation of the β-gal mutants and the possibility of steric hindrance, we tested the plasticity of the system using the FKBP12, rapamycin, FRAP system. In this system FKBP12 binds FRAP only in the presence of rapamycin. We fused FKBP12 and FRAP to the individual β-gal mutants in all possible amino and carboxy terminal orientations and tested their ability to complement one another in the presence of rapamycin. In all orientations tested, complementation was achieved in the presence of rapamycin with at least a 15-fold induction of β-gal activity. This result suggests a high degree of flexibility in the molecules and their ability to complement.

Reportable Outcomes

Constructs made: Retroviral vectors containing truncated ErbB (tErbB) family members and a resistance gene:

pWZL-tErbB1-Δω IRES-Hygromicin
pWZL-tErbB1-Δα IRES-Neomycin
pWZL-tErbB2-Δω IRES-Hygromicin
pWZL-tErbB2-Δα IRES- Neomycin
pWZL-tErbB3-Δω IRES-Hygromicin
pWZL-tErbB3-Δα IRES- Neomycin

tEGFR-Frap-Δα
tEGFR-FKBP12-Δω
FKBP12-Δω
Δω-FKBP12
Frap-Δα
Δα-Frap

Cell lines expressing the above constructs singly and in pairwise combinations:

C2C12-tErbB1-Δω
C2C12-tErbB1-Δα
C2C12-tErbB2-Δω
C2C12-tErbB2-Δα
C2C12-tErbB3-Δω
C2C12-tErbB3-Δα
C2C12-tErbB1-Δω/-tErbB1-Δα
C2C12-tErbB2-Δω/-tErbB2-Δα
C2C12-tErbB3-Δω/-tErbB3-Δα
C2C12-tErbB1-Δω/-tErbB2-Δα
C2C12-tErbB3-Δω/-tErbB1-Δα

tEGFR-Frap-Δα + tEGFR-FKBP12-Δω
tEGFR-Frap-Δα + FKBP12-Δω
tEGFR-Frap-Δα + Δω-FKBP12
tEGFR-FKBP12-Δω + Frap-Δα
tEGFR-FKBP12-Δω + Δα-Frap

Frap-Δα + FKBP12-Δω
Frap-Δα + Δω-FKBP12
Δα-Frap + FKBP12-Δω
Δα-Frap + Δω-FKBP12

Conclusions

Preliminary experiments suggest that this approach has a high likelihood of success. It is the only system to our knowledge that will permit receptor interaction to be monitored at the membrane and new interacting proteins to be identified. Our progress during this year has been highly encouraging and quite substantial.

References

Blakely, B.T., Rossi, F. M.V., Tillotson, B., Palmer, M., Estellés, A. and Blau, H.M. (2000) Epidermal growth factor receptor dimerization monitored in live cells. Nature Biotech. 18:218-222.

APPENDICES

Reprints:

Blakely, B.T., Rossi, F. M.V., Tillotson, B., Palmer, M., Estellés, A. and Blau, H.M. (2000) Epidermal growth factor receptor dimerization monitored in live cells. Nature Biotech. 18:218-222.

Rossi, F.M.V., Blakely, B.T. and Blau, H.M. (2000) Interaction blues: Protein interactions monitored in live mammalian cells by β–gal complementation. Trends in Cell Biology 10:119-122.

Rossi, F.M.V., Blakely, B.T., Charlton, C.A. and Blau, H.M. (2000) Monitoring protein-protein interactions in live mammalian cells by β-galactosidase complementation. In Applications of Chimeric Genes and Proteins. Methods in Enzymology, vol. 328. (J.N. Abelson, S.D. Emr, J. Thorner, eds.) Academic Press, San Diego, pp. 231-251.

Epidermal growth factor receptor dimerization monitored in live cells

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We present a method for monitoring receptor dimerization at the membrane of live cells. Chimeric proteins containing the epidermal growth factor (EGF) receptor extracellular and transmembrane domains fused to weakly complementing β-galactosidase (β-gal) deletion mutants were expressed in cells in culture. Treatment of the cells with EGF-like compounds for as little as 15 s resulted in chimeric receptor dimerization detectable as β-gal enzymatic activity. The dose response of chimeric receptors was ligand specific. β-galactosidase complementation was reversible upon removal of ligand and could be reinduced. Antibodies that block ligand binding inhibited receptor dimerization and β-gal complementation. These results demonstrate that β-gal complementation provides a rapid, simple, and sensitive assay for protein interactions and for detecting and monitoring the kinetics of receptor dimerization.

Keywords: Protein interactions, receptor dimerization, EGF receptor

Dimerization, or higher order oligomerization, of cell surface receptors is often a prerequisite for their activation and signal transduction. For example, EGF receptor homodimerization, as well as its heterodimerization with the related erbB2 receptor, are stabilized by the binding of several EGF-related peptide growth factors. Following ligand binding and dimerization, the receptor undergoes a conformational change that leads to its autophosphorylation and activation of the receptor tyrosine kinase[1–3].

Chemical crosslinking, immunoprecipitation, and fluorescence resonance energy transfer (FRET) have been used extensively to study receptor dimerization. However, biochemical methods require cell disruption and do not preserve all interactions. The yeast two-hybrid system[4] cannot be used for integral membrane proteins. More recently, methods such as FRET analysis of proteins tagged with green fluorescent protein (GFP) and complementation of β-gal and dihydrofolate reductase (DHFR) have made possible the detection of protein interactions in live mammalian cells[5–7].

We have shown previously that complementation of bacterial β-gal, first demonstrated in prokaryotes[8], can be used to detect cytoplasmic protein interactions in mammalian cells[6,9]. When two different, weakly complementing deletion mutants of β-gal, Δα and Δω, are fused to two interacting proteins and expressed in a cell, the interaction of the non-β-gal portions of the chimeric proteins drives β-gal complementation, and the resulting β-gal activity serves as a measure of that interaction. There are several advantageous properties of β-gal complementation: (1) it works in live mammalian cells; (2) it monitors interactions in the cellular compartment in which they normally occur; (3) the signal is amplified by the enzymatic activity of β-gal; (4) the signal can be quantitated by biochemical, histochemical, fluorescent, and chemiluminescent assays; (5) overexpression of complementing protein chimeras can be avoided, as low levels are readily detected[10,11].

Here we report that by using β-gal complementation to monitor membrane protein interactions, dimerization of the EGF receptor can be readily detected using rapid assays amenable to high-throughput screening methods. This system facilitates the study of signal transduction and should be useful for screening for agonists and antagonists of several receptors.

Results

Expression of EGF receptor–β-gal chimeric proteins. The weakly complementing Δα and Δω deletion mutants of β-gal were fused to the extracellular and transmembrane regions of the human EGF receptor to form chimeric receptor molecules (Fig. 1A). Deletion of the cytoplasmic domain of the receptor removes the sequences that lead to a decrease in receptor activity by either internalization or phosphorylation on serine and threonine residues[12]. The use of such a truncated receptor avoided internalization of the chimeric receptors, and the consequent decrease in surface expression, eliminating this variable from analyses of receptor dimerization over time. The two chimeric cDNAs were each cloned into retroviral vectors encoding selectable markers (Fig. 1B) and transduced into C2C12 mouse myoblasts. After selection with G418 and hygromycin, cells were treated with EGF, and β-gal enzyme activity was assayed in live cells using a fluorogenic substrate and the fluorescence-activated cell sorter (FACS). In cells expressing only one of the chimeric molecules, no change in β-gal activity was observed upon addition of EGF, demonstrating that β-gal mutants of one type cannot form an active β-gal enzyme (Fig. 1C, 1D).

In cells expressing both chimeric molecules, EGFR–Δα–β-gal and EGFR–Δω–β-gal, FACS analysis showed that β-gal activity was significantly induced by EGF. In the absence of EGF, the population of transduced cells consisted of a mixture of cells with low and high levels of β-gal activity (Fig. 1E, black curve), which was not unexpected as it is known that the EGF receptor can dimerize in the absence of EGF in cell lines, such as A431, that express high levels of the receptor[13]. Much of the subpopulation that had low β-gal activity underwent a ligand-dependent increase in β-gal activity following stimulation with EGF (Fig. 1E, red curve).

Complementation of β-gal is dependent on the specific interaction of the non–β-gal portions of the chimeric receptor, as coexpression of EGFR–Δω–β-gal with a chimeric protein that contained the

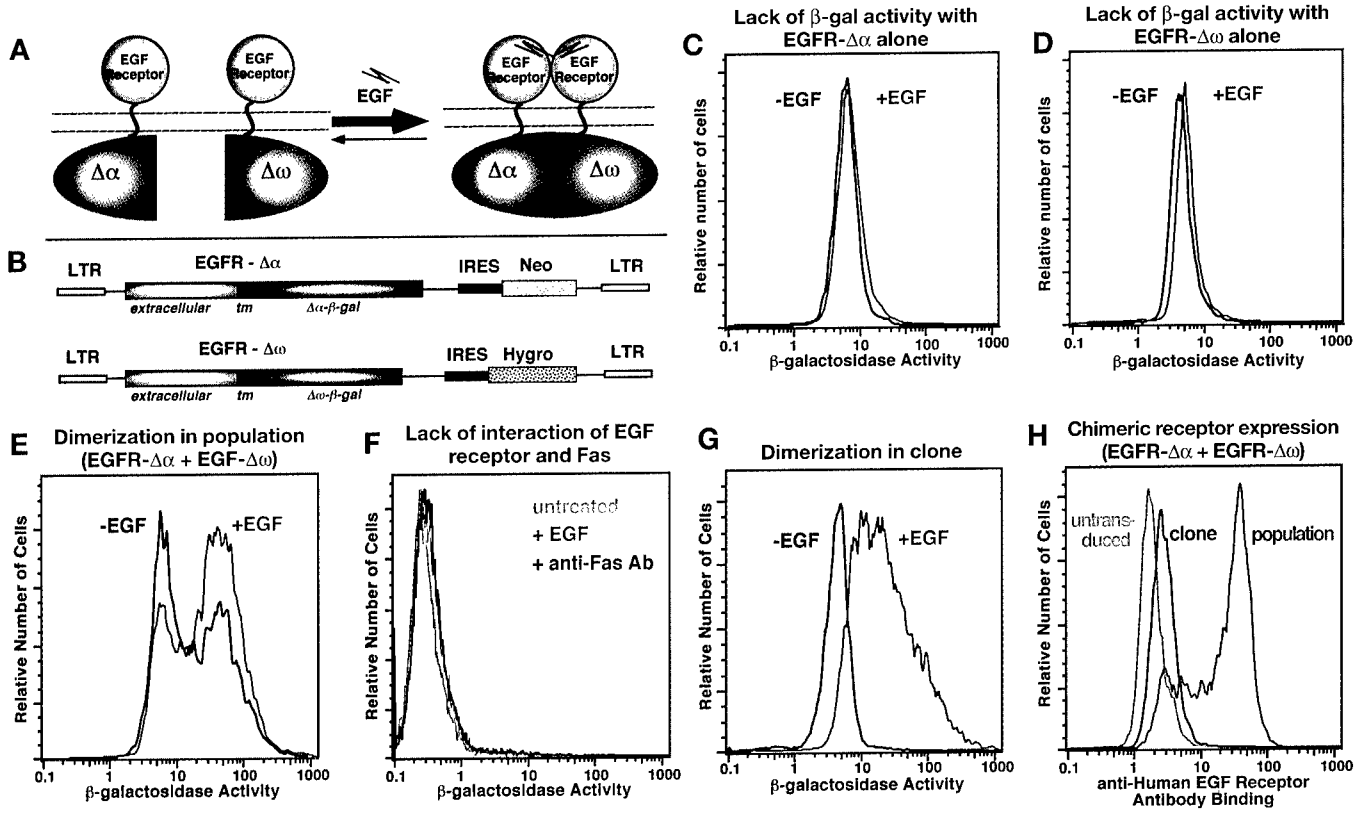

Figure 1. EGF receptor dimerization monitored using β-gal complementation. (A) Two weakly complementing deletion mutants of β-gal linked to the extracellular and transmembrane domains of the EGF receptor to determine whether receptor dimerization, which is stabilized by the addition of EGF, can drive β-gal complementation. (B) The human EGF receptor (EGFR) extracellular and transmembrane domains cloned 5′ to and in frame with the E. coli lacZ deletion mutants Δα and Δω in retroviral vectors expressing neomycin or hygromycin resistance, respectively. (C, D) FACS profiles of β-gal activity (fluorescein fluorescence) in C2C12 cells expressing only one chimeric receptor, either EGFR–Δα–β-gal (C) or EGFR–Δω–β-gal (D), without EGF treatment (black curve) and treated with EGF for 2 h (red curve). (E) β-gal activity in a population of C2C12 cells expressing both chimeric receptors and treated with EGF for 2 h (red curve). Untreated cells are shown by the black curve. (F) Coexpression of a Fas–Δα–β-gal chimera with the truncated EGFR–Δω–β-gal chimera in NIH-3T3 cells. β-gal activity is shown for cells treated with EGF for 2 h (red curve) or treated with the activating anti-Fas antibody Jo2 for 6 h (100 ng/ml; blue curve). Untreated cells are shown by the green curve. (G) β-gal activity in a clone derived from the cells shown in (E) treated with EGF for 2 h (red curve). Untreated cells are shown by the black curve. (H) FACS profiles of an immunofluorescence assay for the expression of the chimeric receptor in the population of cells shown in (E) (red curve), in the clone shown in (G) (blue curve), and in untransduced cells (green curve)

membrane receptor Fas (CD-95)[14] fused to Δα–β-gal did not yield β-gal activity when either EGF or an activating anti-Fas antibody was added to cells (Fig. 1F). This was not a result of the inability of Fas–β-gal chimeras to dimerize and complement, as Fas receptor activation with the anti-Fas antibody resulted in detectable β-gal activity when Fas–Δα–β-gal and Fas–Δω–β-gal were coexpressed

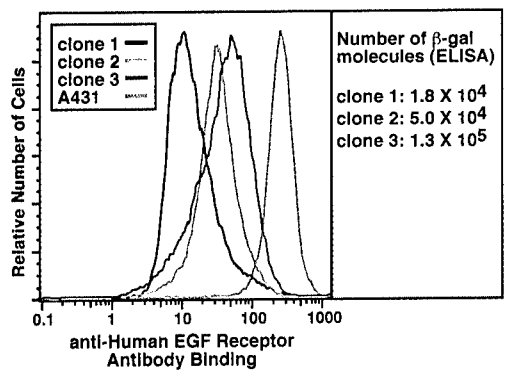

Figure 2. Chimeric receptor expression compared to A431 cells. A431 cells and three clones expressing chimeric receptors were immunofluorescently labeled and assayed by FACS as in Fig. 1H. The same three clones were analyzed by ELISA with an anti-β-gal antibody, and the number of chimeric receptors was determined from a standard curve using purified β-gal protein.

(A.E. and H.M.B., in preparation). These results demonstrate that the complementation of β-gal monitors but does not drive the interaction of the chimeric proteins.

Cells transduced with EGFR–Δα–β-gal and EGFR–Δω–β-gal expressed a broad range of concentrations of the chimeric receptor (Fig. 1H, red curve), which might account for the range in β-gal activity observed in the population. Single cells were isolated using either the FACS or limiting dilution to select for low background amounts of β-gal activity in the absence of EGF and increased amounts of β-gal activity in the presence of EGF. Three dozen clones were tested for ligand-dependent β-gal activity, one of which is shown (Fig. 1G). Clones that had low β-gal activity in the absence of EGF and exhibited a several-fold increase in β-gal activity in the presence of EGF had lower levels of chimeric receptor expression than much of the population (Fig. 1H, blue curve).

We analyzed chimeric receptor expression in three clones that showed ligand-dependent β-gal activity (Fig. 2). Analysis by FACS shows that these clones expressed different amounts of the receptor, but all of them expressed significantly less receptor than the A431 epithelial carcinoma cell line, which is known to express about 2.6×10^6 receptors per cell[15]. The same clones were analyzed by enzyme-linked immunosorbent assay (ELISA) using antibodies to bacterial β-gal and compared to a standard curve generated using purified β-gal protein. These results show that the clones express 1.8×10^4–1.3×10^5 β-gal molecules (chimeric receptors) per cell (Fig. 2), within the range for EGF receptor expression observed in normal cells (up to 3

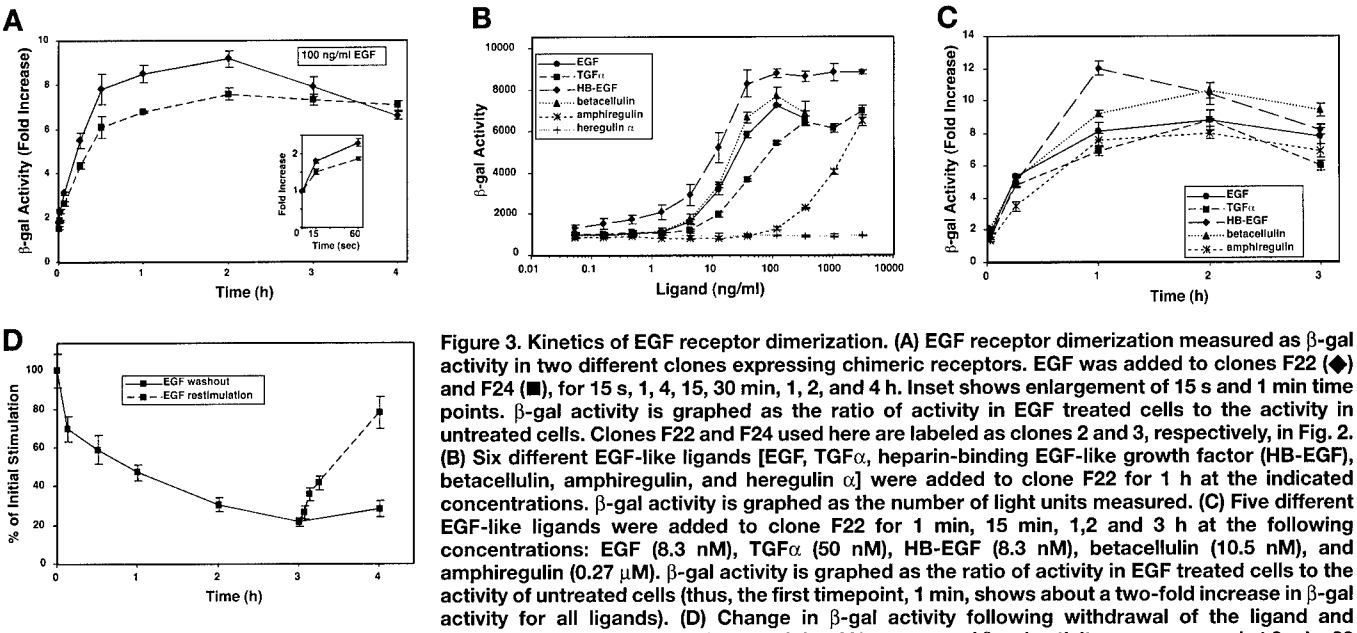

Figure 3. Kinetics of EGF receptor dimerization. (A) EGF receptor dimerization measured as β-gal activity in two different clones expressing chimeric receptors. EGF was added to clones F22 (◆) and F24 (■), for 15 s, 1, 4, 15, 30 min, 1, 2, and 4 h. Inset shows enlargement of 15 s and 1 min time points. β-gal activity is graphed as the ratio of activity in EGF treated cells to the activity in untreated cells. Clones F22 and F24 used here are labeled as clones 2 and 3, respectively, in Fig. 2. (B) Six different EGF-like ligands [EGF, TGFα, heparin-binding EGF-like growth factor (HB-EGF), betacellulin, amphiregulin, and heregulin α] were added to clone F22 for 1 h at the indicated concentrations. β-gal activity is graphed as the number of light units measured. (C) Five different EGF-like ligands were added to clone F22 for 1 min, 15 min, 1,2 and 3 h at the following concentrations: EGF (8.3 nM), TGFα (50 nM), HB-EGF (8.3 nM), betacellulin (10.5 nM), and amphiregulin (0.27 μM). β-gal activity is graphed as the ratio of activity in EGF treated cells to the activity of untreated cells (thus, the first timepoint, 1 min, shows about a two-fold increase in β-gal activity for all ligands). (D) Change in β-gal activity following withdrawal of the ligand and restimulation. Clone F22 was treated for 1 h with EGF, rinsed twice with media containing 2% serum, and β-gal activity was measured at 8 min, 30 min, 1, 2, 3 and 4 h thereafter. After 3 h in the absence of EGF, some samples were again treated with EGF for 4, 8, and 15 min and 1 h. β-gal activity is graphed as the ratio of activity in EGF treated cells to the activity of untreated cells normalized to the value for the initial 1 h treatment which was set to 100%. β-gal activity was measured in triplicate (B and C) or quadruplicate (A and D) samples; error bars indicate standard deviation.

× 10^5 receptors/cells)[16,17]. Thus, β-gal activity is a useful measure of ligand-dependent receptor dimerization without greatly overexpressing the chimeric receptor.

Dimerization kinetics and ligand specificity assayed by β-gal complementation. Although the FACS permits the analysis of live single cells, it is cumbersome to analyze more than 100 samples within a few hours. However, chemiluminescence assays allow rapid analysis of thousands of samples on multiwell culture plates within ~1 h. Using such an assay, we analyzed dimerization kinetics of several ligands at several concentrations. Dimerization, expressed as the fold increase in β-gal activity, could be detected with EGF treatments as short as 15 s (Fig. 3A, inset). With longer exposure to EGF, dimerization continued to increase rapidly for up to 2 h (Fig. 3A). This time course is in good agreement with previous data on EGF receptor kinetics including ligand binding, receptor activation, and substrate phosphorylation, which indicate that the receptor responds to ligand within minutes[18,19].

Six EGF-like growth factors were used to test for the specificity of receptor dimerization and β-gal complementation. The dose/response curves for EGF-like ligands differ (Fig. 3B), but show that dimerization increased with increasing concentrations of the following ligands: EGF, tumor growth factor (TGF)-α, heparin-binding EGF-like growth factor, amphiregulin, and betacellulin. The dose/response curve for amphiregulin demonstrated the significantly lower affinity of amphiregulin relative to the other ligands for the EGF receptor[20]. As expected, dimerization was not induced by the EGF-like growth factor heregulin α, which is known to activate members of the EGF receptor family, but not the EGF receptor itself[20]. The rate of dimerization was measured by β-gal complementation using the concentrations of each ligand found to elicit a maximum response (Fig. 3B) and was similar for all activating ligands (Fig. 3C).

Following removal of the ligand from the medium, EGF receptor dimerization decreased (Fig. 3D). Cells were rinsed to remove free ligand following a 1-h treatment with EGF, at which time point β-gal activity was still increasing (see Fig. 3A). Dimerization began to decline shortly after removal of the ligand. Restimulation of receptor dimerization was induced by the reintroduction of EGF into the medium (Fig. 3D).

Inhibition of receptor dimerization. No significant increase in dimerization was observed in cells treated with both EGF and either monoclonal antibody 225 (Fig. 4) or 528 (not shown), both of which are known to block binding of EGF to the human receptor[21]. Neither an anti-hemagglutinin (anti-HA) antibody or another antibody to the EGF receptor (antibody 455), inhibited EGF receptor dimerization. Monoclonal antibody 13A9 had previously been shown to inhibit TGF-α binding, but not EGF binding, to the receptor[22]. Another study described the surprising finding that although EGF binding and EGF-dependent activation of the EGF receptor occurred in the presence of antibody 13A9, EGF receptor dimerization could not be detected in the presence of this antibody either by immunoprecipitation, by density gradient centrifugation, or by FRET (ref. 23). As expected, we found that antibody 13A9 did inhibit TGF-α induced dimerization. However, in contrast to the previous findings, EGF-stimulated receptor dimerization in the presence of 13A9 was readily detected as β-gal activity (Fig. 4). The same results were obtained with the Fab fragment of anti-

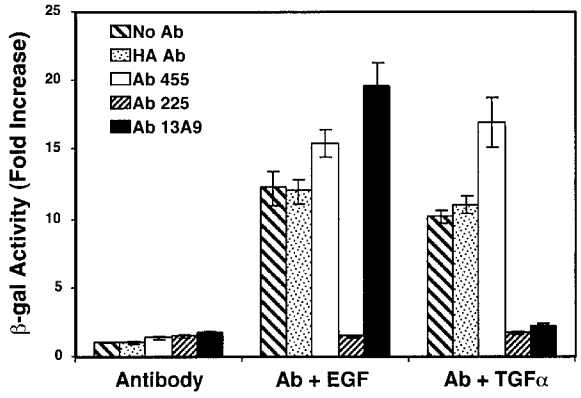

Figure 4. Antibodies which inhibit ligand binding inhibit dimerization. β-gal activity assayed in cells (clone F22) preincubated with monoclonal antibodies (anti-HA and anti-EGF receptor antibody 455, which does not inhibit ligand binding; antibody 225, which inhibits ligand binding; or 13A9, which inhibits TGF-α but not EGF binding) at 25 ng/ml for 5 min and then treated with EGF (100 ng/ml) or TGF-α (300 ng/ml) for 1 h. β-gal activity was measured in quadruplicate samples and is graphed as a ratio of the activity in EGF treated cells relative to untreated cells. Error bars indicate standard deviation.

body 13A9 for both TGF-α and EGF (data not shown).

Because 13A9 does not block receptor activation by EGF, it has been suggested that EGF receptor activation could occur in the absence of dimerization[23]. An alternative explanation presented for this apparent paradox is that 13A9 destabilizes or perturbs receptor dimerization to a degree that prevented detection of dimers by the above methods, but allowed sufficient interaction so that activation occurred[23]. Here we show that receptor dimerization in the presence of antibody 13A9 can in fact be detected if the β-gal complementation assay is used, a result that is in agreement with accepted models in which dimerization precedes receptor activation.

Discussion

Critical to the use of our method for monitoring protein interactions is that the kinetics of the β-gal activity reflect the properties of the interacting non-β-gal portions of the chimeric protein pairs. This appears to be the case based on studies of two different protein pairs. Previously, we showed that in cells expressing chimerae of rapamycin-binding proteins and complementing β-gal mutants, β-gal complementation was specifically induced by addition of rapamycin. β-gal activity began to increase slowly after 30 min, and continued to increase over the subsequent 24h. As shown here, EGF receptor dimerization was detectable as β-gal activity with EGF treatments of as little as 15 seconds and continued to increase, reaching peak levels within one or two hours. Thus, the kinetics of these two distinct sets of interacting proteins monitored by the complementation of the same β-gal mutants differed markedly from one another. Furthermore, β-gal complementation was specific and no enzyme activity was detected if the complementing mutants were linked to non-interacting membrane proteins such as the EGF receptor and Fas. Taken together, these data indicate that β-gal complementation serves to monitor, not to drive, the chimeric protein interactions in each case.

Receptor dimerization has typically been analyzed by in vitro methods such as chemical crosslinking and immunopurification, followed by gel electrophoresis[24,25]. Recently, EGF receptor dimerization has been analyzed in single cells by FRET[13]. Analyses of interactions of proteins conjugated to fluorophores within the cell or cell membrane by FRET have been limited by the difficulty of labeling these molecules in vitro and introducing them at sufficiently high concentrations. This problem has been overcome by the elegant method developed by Tsien and colleagues in which chimeric proteins containing GFP are expressed in cells[5]. By using two different GFP mutants that emit at different wavelengths, FRET analysis of protein interactions is possible[26]. However, unlike the β-gal activity produced by β-gal complementation, the GFP signal cannot be enzymatically amplified. As a result, achieving a detectable signal is limited to some extent by the need to achieve a relatively high level of expression of the chimeric protein.

Since our initial description of the β-gal complementation assay[6], complementation of another protein, DHFR, has been used to monitor protein interactions[7,27]. Complementation of DHFR is assayed by measuring the binding of a fluorescently labeled inhibitor, methotrexate. As with GFP chimeras, the DHFR signal is not enzymatically amplified, and requires a sufficiently high expression level for detection. The DHFR system has been used primarily in cells lacking endogenous DHFR, and has not been used for quantitative assays in mammalian cells that express endogenous DHFR. The enzymatic signal amplification characteristic of the complemented β-gal, as well as the availability of sensitive substrates and rapid assays, enables complementation of this enzyme to be detected without grossly overexpressing the proteins, thus avoiding potential perturbation of normal cellular mechanisms. As shown here, the β-gal complementation system works well without overexpressing the chimeric proteins at high levels, although clearly some dimerization events do not lead to complementation and signal generation (e.g., homodimers between EGF receptors linked to the same β-gal mutant). If a signal were not detected with moderate expression levels, this study would not have been successful as overexpression of the EGF receptor leads to ligand-independent dimerization (Fig. 1). An assay system that does not require overexpression will be invaluable for the study of other proteins such as Fas, as overexpression of Fas can lead to apoptosis.

Because the active bacterial β-gal is known to be a tetramer, it was not obvious that a molecule that requires both complementation and oligomerization to form an active enzyme would be useful in monitoring the dimerization of a linked protein. However, it has been shown that in the formation of an active complemented β-gal protein, the assembly of the complex is relatively rapid, and the rate-limiting step is the conformational changes that occur after oligomerization of the subunits[28,29]. Our data showing that EGF receptor dimerization can be detected with ligand treatments as short as 15 s using β-gal complementation provides support for this model in eukaryotic cells.

Because the cells used here express an endogenous mouse EGF receptor, which can bind ligand in the presence of these human receptor-specific antibodies, the antibody inhibition results show that dimerization of the chimeric human receptor can be blocked when the endogenous receptor is dimerized and activated. Furthermore, evidence that the endogenous receptor is not required for chimeric receptor dimerization is provided by expressing the same constructs in fibroblasts (3T3 clone 2.2), which lack an endogenous mouse EGF receptor but yield similar results to the cells shown here (data not shown). These findings indicate that β-gal complementation is driven by specific interactions of the chimeric receptor, not by a generalized clustering of EGF receptor molecules in the cell.

In conclusion, β-gal complementation provides a rapid and sensitive method for monitoring receptor dimerization at the membrane of live cells. Once the appropriate fusion construct is generated, populations of cells or clone, if necessary, can be obtained and tested within a few weeks. Complementation of β-gal may also provide a screen for novel interacting proteins in a mammalian "two-hybrid" assay in which the protein complexes are monitored in the cell compartment in which they normally occur.

Since complementation occurs in situ in intact live cells, the effect of agents, such as antibody 13A9, on receptor dimerization can be more accurately detected than with previously available methods. This approach provides a potent tool for high throughput screeening for pharmacological agents that can bind to receptors and act as either agonists or antagonists. The blocking antibodies used here have also been shown to inhibit tumor cell growth in vitro and similar reagents that act on the related erbB2 receptor have been used to treat cancer[19,30]. Such potentially therapeutic agents can be rapidly screened using the type of system presented here. That such assays are possible is clear from preliminary results in our laboratory that indicate that β-gal complementation can detect heterodimerization of erbB2 and EGF receptor.

Experimental protocol

Construction and expression of chimeric receptors. The sequence coding for the extracellular and transmembrane domains of the human EGF receptor (amino acids 1–655) was amplified by polymerase chain reaction (PCR). Although this fragment retains Thr654, which is a site of protein kinase C (PKC) phosphorylation, Arg656 and Arg657 are removed, destroying the consensus PKC recognition sequence. The amino acid sequence beginning with Thr654 is Thr-Leu-Glu-Ser-Met, where the methionine is the beginning of the β-gal sequence, and the glutamic acid and serine are generated by the junction sequence and are not native to either EGF or β-gal. The resulting fragment was cloned into the pWZL-Δα and pWZL-Δω vectors, in frame with the Δα and Δω β-gal mutants[6,9]. Virus was produced and cells were

infected as previously described[6,9]. Cells were selected in 1 mg/ml G418 and 1 mg/ml hygromycin, and were maintained in 400 μg/ml of each antibiotic. Populations of cells were ready for analysis within two weeks of infection. Clones were obtained by either cell sorting or limiting dilution. Full-length murine Fas cDNA was similarly cloned into the above vector in frame with the Δα and Δω β-gal mutants.

FACS analysis. Cells were treated with mouse salivary gland EGF (Sigma, St. Louis, MO) at 100 ng/ml. Following all treatments fluorescein di-β-D-galactopyranoside (FDG; Molecular Probes, Eugene, OR) was immediately loaded into the cells by hypotonic shock as described[31,32]. Cells were then kept on ice until analysis on the FACS, which occurred 1–2 h after trypsinization. The chimeric receptor was detected by immunofluorescence using a mouse monoclonal antibody specific to the extracellular domain of the human EGF receptor (antibody R1, Dako, Carpinteria, CA, and Calbiochem, La Jolla, CA). Data shown here as FACS profiles in Figure 1 were adjusted for autofluorescence using autofluorescence compensation[33]. Mean fluorescence data for β-gal activity were adjusted for both autofluorescence and endogenous mammalian β-gal activity by subtracting the mean fluorescence of untransduced cells loaded with FDG substrate.

Chemiluminscent analysis of β-gal activity. Triplicate or quadruplicate samples of cells were plated at 10,000/well in 100 μl volume in white 96-well plates (Corning Costar, Acton, MA). After 16–24 h, the spent media was removed and replaced with media containing growth factors, or in some cases, antibodies. Cells were lysed by addition of GAL-Screen substrate (Tropix PE Biosystems, Bedford MA, buffer B formulation), and the plates were incubated at 27°C for 40 min. Luminescence was measured in a Tropix TR717 luminometer, or in a Wallac MicroBeta Plus.

Analysis of kinetics and inhibition of dimerization. Recombinant human growth factors were obtained from Life Technologies, Rockville, MD (EGF), R&D Systems, Minneapolis, MN (TGF-α in Figure 3, heparin-binding EGF-like growth factor, betacellulin, amphiregulin, heregulin α), and Sigma (EGF and TGF-α in Figs 3D and 4). Mouse monoclonal antibodies used were clone 528, clone 225, clone 450, and clone R1 (Calbiochem) and antibody 13A9 (gift of Genentech). Anti-HA mouse monoclonal antibody 12CA5 (Roche Molecular Biochemicals) was used as a negative control. Antibodies were added to cells for 5 min at room temperature before the addition of growth factor, at which point cells were returned to 37°C.

ELISA detection of β-gal chimeric proteins. Cells were lysed in lysis buffer [50 mM HEPES pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 0.01% thimerosol, and a protease inhibitor cocktail containing AEBSF, pepstatin A, E-64, bestatin, leupeptin, and aprotinin (Sigma)] and lysates were centrifuged to remove debris. ELISA plates were coated with 1 μg/ml mouse anti-β-gal antibody 5B88 (Life Technologies) in 4 μg/ml BSA in PBS and then blocked for 2 h (Superblock, Pierce Chemical, Rockford, IL). Cell lysates were diluted in lysis buffer with 0.2% BSA and 0.05% Tween-20 and 100 μl of diluted lysate representing 1×10^4 to 1×10^5 cells was added to each well. To generate a standard curve, purified *Escherichia coli* β-gal protein (Sigma) was diluted in the same buffer to final concentrations of 1.2 pg–20 ng per well. After 30 min, wells were washed five times with 0.2% Tween-20 in PBS. To each well were added 100 μl of biotinylated mouse anti-β-gal antibody GAL-13 (Sigma) diluted 1:5,000 in 0.2% BSA, 0.05% Tween-20 in PBS; these were incubated for 30 min at room temperature, and then washed as before. To each well were then added 100 μl of streptavidin–alkaline phosphate conjugate (Tropix) diluted 1:5,000 in 0.2% BSA, 0.05% Tween-20 in PBS; these were incubated for 25 min at room temperature, and then washed as before. Finally, 100 μl of CSPD-Sapphire II chemiluminescent substrate (Tropix) were added to each well, and after 30 min, the luminescence was measured on a Tropix TR717 luminometer.

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Interaction blues: protein interactions monitored in live mammalian cells by β-galactosidase complementation

Fabio M.V. Rossi, Bruce T. Blakely and Helen M. Blau

In mammalian cells, protein–protein interactions constitute essential regulatory steps that modulate the activity of signalling pathways and many other intracellular processes. Although efficient genetic methods for identifying interacting partners exist and have been successfully applied by a number of laboratories, there has been a need for a technique that allows protein interactions to be monitored in real time in the cellular compartment in which they normally take place. The β-galactosidase-based intracistronic complementation methodology described here is the first technology that might fulfil these requirements and can be applied to live mammalian cells. In addition, it holds promise for applications in high-throughput screens for agonists and antagonists of specific interactions and for the development of a 'mammalian two-hybrid' screen for novel protein partners.

Cell physiology and development is controlled by well-regulated cascades of protein–protein interactions. For example, the activation and subsequent auto-phosphorylation of growth-factor receptors is often dependent on their ligand-induced homo- or heterodimerization[1]. Such protein interactions result, in turn, in the creation of docking sites for downstream components of the relevant signalling pathways and thus in additional protein–protein interactions[2]. Several systems have been developed for identifying and studying protein–protein interactions, including the yeast two-hybrid system[3,4], the split-ubiquitin system[5,6], the Sos-recruitment system[7,8] and dihydrofolate reductase (DHFR) complementation[9,10]. As shown in Table 1, each of these systems has inherent advantages, as well as disadvantages, including lack of utility in mammalian cells or with membrane proteins, or lack of rapid quantitative analysis of the interaction owing to the absence of signal amplification, indirect readouts or other assay limitations.

We have developed a novel assay for monitoring protein–protein interactions based on intracistronic β-galactosidase complementation. There are several advantageous properties of the intracistronic β-galactosidase complementation method:

- it works in live mammalian cells;
- it monitors interactions in the compartment in which they normally take place (e.g. membrane or cytoplasm);
- rapid sensitive assays are available that are amenable to high-throughput screening methods;
- it provides a quantitative readout, allowing the monitoring of interaction kinetics;
- it provides signal amplification, allowing physiological interactions to be monitored in the absence of overexpression.

Properties of β-galactosidase intracistronic complementation for monitoring protein–protein interactions

Intracistronic β-galactosidase complementation is a phenomenon whereby two mutants of the bacterial enzyme β-galactosidase that harbour inactivating mutations in different crucial domains are capable of recreating an active enzyme by sharing their intact domains[11,12]. It has long been known that, in *Escherichia coli*, specific mutants can complement one another more or less efficiently, depending on the nature of the mutations[13]. We have shown that the same holds true in mammalian cells[14]. Our protein-interaction detection method capitalizes on the expression of low levels of chimeric proteins incorporating weakly complementing β-galactosidase mutants. β-galactosidase activity is recreated only when physical interaction of the mutants is forced by the non-β-galactosidase components of the hybrids. Under these conditions, the complementation of β-galactosidase mutants does not drive, but rather monitors, the interaction of other proteins.

To monitor the interaction between two proteins, each of the proteins is

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TABLE 1 – ADVANTAGES AND DISADVANTAGES OF SOME SYSTEMS FOR ANALYSING PROTEIN–PROTEIN INTERACTIONS

System	Works in mammalian cells?	Works with membrane proteins?	Pros	Cons	Refs
β-galactosidase complementation	Yes	Yes	Allows real-time measurements of interactions. Owing to enzymatic amplification of the signal, works as a tracer in the absence of overexpression.	Because it relies on re-assembly of a complex enzyme, it is likely to be subject to steric constraints.	16
Yeast two-hybrid	No (yeast)	No	Widely used and very successful in detecting novel interaction partners of a given test protein.	Has not been successfully used in mammalian cells. Requires nuclear localization of the hybrid proteins, thus it cannot be used with membrane proteins. Indirect readout limits the kinetic analysis of interactions.	3
Sos-recruitment system	No (yeast)	No	Good sensitivity and direct selection for positive colonies based on growth. Likely to be independent of steric constraints.	Cannot be used in mammalian cells or with transmembrane proteins. Indirect readout limits the kinetic analysis and subcellular localization of interactions.	7, 8
DHFR complementation system	Yes	Yes	In DHFR mutant cells, allows direct selection of positive clones based on growth.	No enzymatic amplification in cells containing wild-type DHFR, thus requires overexpression of the test proteins. Not yet shown to allow quantitative measurements of interactions in wild-type DHFR cells. Likely to be subject to steric constraints.	9, 10
Split-ubiquitin system	Yes	Yes	Good sensitivity owing to transcription-mediated signal amplification.	Indirect readout limits the kinetic analysis and subcellular localization of interactions. Likely to be subject to steric constraints.	5, 6

fused to one of a pair of β-galactosidase mutants (Fig. 1), and the two fusion proteins are expressed at low levels in mammalian cells. The interaction between the two test proteins and the consequent juxtapositioning of the mutants leads to complementation and an increase in β-galactosidase activity. For use with this system, we have developed a specific pair of β-galactosidase deletion mutants (Δα and Δω; Fig. 2) that display a low level of spontaneous complementation when coexpressed in mammalian cells[14]. Low levels of the chimeric proteins are expressed in order to avoid perturbing the physiological balance of the cell and further reduce non-specific interactions, thereby minimizing basal β-galactosidase activity. This is achieved by introducing single copies of constructs into cells by using limiting dilution of the two retroviruses and by expressing the chimeric protein and a selectable marker from the same bi-cistronic messenger RNA[15,16].

A particularly important characteristic of the intracistronic complementation method is that the enzyme that provides the readout of the assay is linked covalently to the interacting proteins. Thus, the signal is generated directly in the subcellular compartment in which the interaction takes place. Using this system, we have been able to monitor ligand-induced interactions between two cytoplasmic proteins (FKBP12 and the FRB domain of FRAP)[16,17], between a cytoplasmic protein and a transmembrane receptor (FADD and FAS) (Ref. 18; A. Estellés *et al.*, unpublished), and the formation of homodimers and higher-order complexes between transmembrane proteins (EGF receptor and FAS, respectively)[1,19,20].

Crucial to the future broad application of this assay was the finding that the kinetics of appearance of β-galactosidase activity in response to a particular inducer varied and reflected the properties of the protein pairs tested. When the dimerization of chimeric epidermal growth factor receptor (EGF-R)–β-galactosidase constructs was monitored, an increase in β-galactosidase activity was detectable within 15 seconds after treatment with EGF, which peaked within one to two hours[20]. By contrast, in the case of the FKBP12–FRAP interaction, β-galactosidase activity was first detected 0.5 hours after the inducer, rapamycin, was added to the culture medium, and this activity increased steadily over the subsequent 12 hours, in good accordance with previously published results[16,17,21]. We conclude therefore that β-galactosidase activity reflects the characteristics of the interactions being monitored rather than the rate of the β-galactosidase

complementation reaction. This was not a foregone conclusion since the reconstitution of β-galactosidase activity by intracistronic complementation is thought to require partial refolding of the mutants and subsequent assembly of the tetrameric active enzyme[22,23]. In theory, a considerable amount of time might be required for the development of a detectable β-galactosidase signal. Our experiments suggest that such a lag is on the order of seconds and should therefore be negligible for most applications.

Since β-galactosidase has been extensively used as a marker gene for the past 30 years, a number of diverse assays are available to detect its activity. The 'complemented' β-galactosidase can be detected by histochemistry with both the traditional chromogenic substrate X-gal and a more sensitive fluorogenic substrate (Fluor-X-gal)[14,24]. These microscopic detection methods are useful for the visualization of the enzyme within cells. Sensitive quantitation of complemented β-galactosidase can be achieved by biochemical assays of cell lysates with commercially available chemiluminescence substrates (e.g. the GAL-Screen β-galactosidase detection kit from Tropix). Such assays can be readily adapted to a multiwell format, allowing a rapid test of the effect of a variety of pharmacological and biological agents on the protein interactions of interest. For monitoring enzyme activity in live cells, another sensitive and quantitative method uses the fluorogenic substrate fluorescein di-β-D-galactopyranoside (FdG), which is detectable by flow cytometry[25]. FdG can be introduced into living cells by hypotonic loading, which does not affect cell viability, and the fluorescent cleavage product of the reaction catalysed by β-galactosidase (free fluorescein) remains within the cells, as it is unable to cross the plasma membrane.

A great advantage of the β-galactosidase complementation methodology is that it only requires a low level of expression of the chimeric proteins to generate a detectable signal upon interaction. In the case of the EGF receptor, the best results were obtained with cells that expressed levels of the chimeric proteins comparable with wild-type endogenous proteins[20]. Thus, β-galactosidase complementation can be used under conditions that should not alter the balance of cellular proteins or lead to nonphysiological results.

Potential applications and future directions

Intracistronic complementation assays are easily adapted to work in a multiwell format. Furthermore, some of the assays for detecting β-galactosidase activity can be fully automated. Thus, the most straightforward and immediate application of this method is likely to be in high-throughput screening of combinatorial chemical libraries for compounds that can either block or induce a specific protein–protein interaction. This method is particularly well suited for monitoring interactions between membrane proteins such as receptors, between receptors and cytoplasmic components of the downstream signalling pathway or between cytoplasmic proteins. For example, a chemical library could be screened for compounds that inhibit dimerization (and consequent activation) of the EGF receptor or related family members such as ErbB2, which

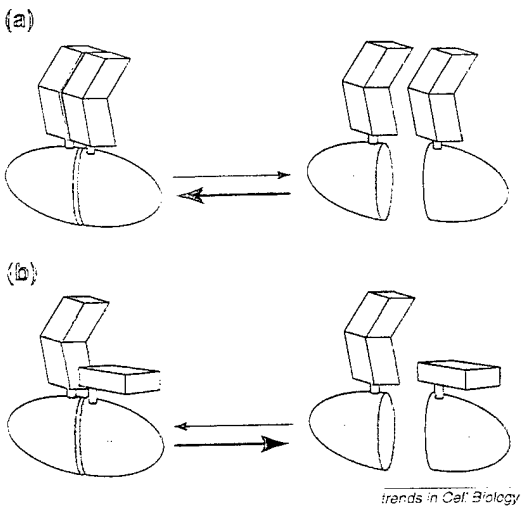

FIGURE 1

Schematic of experimental hypothesis. (a) When the Δα and Δω β-galactosidase mutants are fused to proteins that heterodimerize (represented by the yellow and orange shapes), their association in the active enzyme conformation is favoured. (b) When the two proteins fused to Δα and Δω cannot interact to form a complex, the formation of active β-galactosidase is not favoured. The reconstitution of β-galactosidase activity is dependent on the interaction of the non-β-galactosidase components of the chimeric proteins and allows it to monitor the kinetics of the interaction.

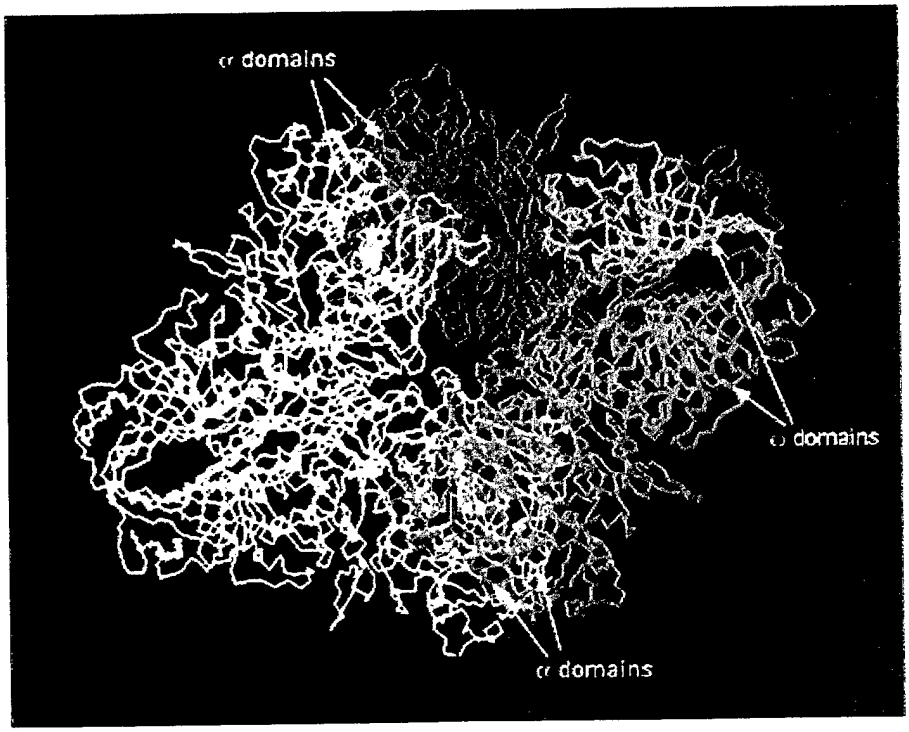

FIGURE 2

Three-dimensional structure of β-galactosidase in its active tetrameric form. Each monomer is represented in a different colour (green, light blue, cyan and dark blue). Two α domains are in red and two in yellow. The two ω domains on the right-hand side of the molecule are represented in red and orange, respectively. Note the extensive intramolecular contacts of the α and ω domains with the central portion of each monomer. In the absence of a forced juxtapositioning of the Δα and Δω mutants, these interactions are likely to sequester the ω and α domains and render them unavailable for complementation.

might thus have anti-neoplastic activity in a subset of human carcinomas, such as tumours of the breast[26].

The ability of intracistronic complementation to monitor interactions in the absence of overexpression might make it applicable to mapping protein interactions directly in transgenic animals. The fusion point between test proteins and β-galactosidase mutants that function best *in vitro* could be recreated *in vivo* by knock-in of Δα and Δω in frame with the endogenous gene. Animals carrying both transgenes should develop β-galactosidase activity only in those cells in which the endogenous proteins are expressed and actually interact. To date, the detection of mRNA and protein localization has been possible in developing organisms. Histochemical detection of β-galactosidase complementation would extend these findings in animal models by allowing the mapping of specific protein interactions in single cells both during embryogenesis and in specific disease states.

Another exciting potential application of intracistronic complementation is the development of a 'mammalian two-hybrid system'. This possibility stems from the ability to use flow-cytometry-based techniques to isolate β-galactosidase-positive cells without compromising their viability. In theory, a chimeric library obtained by fusing random cDNAs to one of the β-galactosidase mutants could be constructed. This library could then be screened for gene products that interact with a given 'bait' protein fused to the complementary β-galactosidase mutant. Such a screen would be unique in that it could be performed in cultured mammalian cells, allowing the detection of interactions that need to be facilitated by an endogenous protein or that only take place in a specific cellular compartment. Furthermore, unlike the yeast two-hybrid system, the readout of the assay would not be dependent on nuclear localization of the interacting partners, allowing this method to be used to isolate partners of membrane-linked proteins.

In theory, the detection of protein–protein interactions by intracistronic complementation could be hindered by steric constraints that prevent formation of an active enzyme. Given two known polypeptides, a range of chimeric proteins could be generated *in vitro* followed by selection of those that display the best characteristics. This limitation might preclude detection of certain interactions in screens of cDNA libraries, in which the cDNA–β-galactosidase fusion points are necessarily generated at random and cannot be predicted or individually optimized. Studies to overcome these problems are under way – for example, testing the insertion of flexible linkers of different length between the test protein and β-galactosidase.

Concluding remarks

Clearly, the intracistronic β-galactosidase complementation method allows monitoring of protein–protein interactions in real time, in live cells, as shown by our analysis of protein interactions involved in cell signalling. Although it is still in the early stages of its development, high-throughput screens for antagonists of specific interactions can be readily envisioned at this time. Improvements will certainly be forthcoming, as well as a deeper understanding of the underlying molecular mechanisms. *In vivo* applications in developing transgenic mice are ongoing. In addition, the development of a 'mammalian two-hybrid system' might soon become a reality.

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[15] Monitoring Protein–Protein Interactions in Live Mammalian Cells by β-Galactosidase Complementation

By FABIO M. V. ROSSI, BRUCE T. BLAKELY, CAROL A. CHARLTON, and HELEN M. BLAU

Introduction

Conditional protein–protein interactions are critical to a vast number of intracellular regulatory mechanisms. A case in point is the activation and autophosphorylation of growth factor receptors, which is often determined by the ligand-dependent induction of receptor dimerization.[1] Docking sites on the activated receptor become accessible to downstream components of the relevant signaling pathways, leading to further protein–protein interactions.[2] Although efficient genetic methods to identify interacting partners exist and have been successfully applied by a number of laboratories, a technique that allows the monitoring of interactions in real time in the cellular compartment in which they normally take place would extend the types of interactions that could be studied. The β-galactosidase-based intracistronic complementation methodology described here is the first technology that fulfills these requirements and can be applied to live mammalian cells.

The novel β-galactosidase (β-Gal) complementation assay for monitoring protein–protein interactions in intact mammalian cells has many advantages (Fig. 1). Unlike the yeast two-hybrid assay,[3] this assay is independent of transcription. Instead, protein interactions are monitored directly *in situ*. Because it is based on the detection of an enzymatic activity, it provides amplification of the signal. Intracistronic β-Gal complementation, first described in prokaryotes by Jacob and Monod more than 30 years ago,[4,5] is a phenomenon whereby two mutants of the bacterial enzyme β-Gal that have inactivating deletions in different critical domains are capable of recreating an active enzyme by sharing their intact domains.[6] It has long been known in *Escherichia coli* that specific mutants can complement each

[1] A. Ullrich and J. Schlessinger, *Cell* **61,** 203 (1990).
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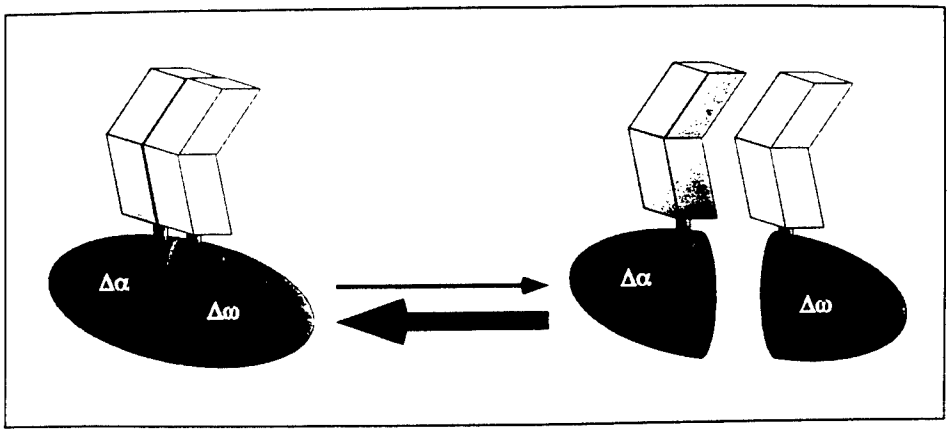

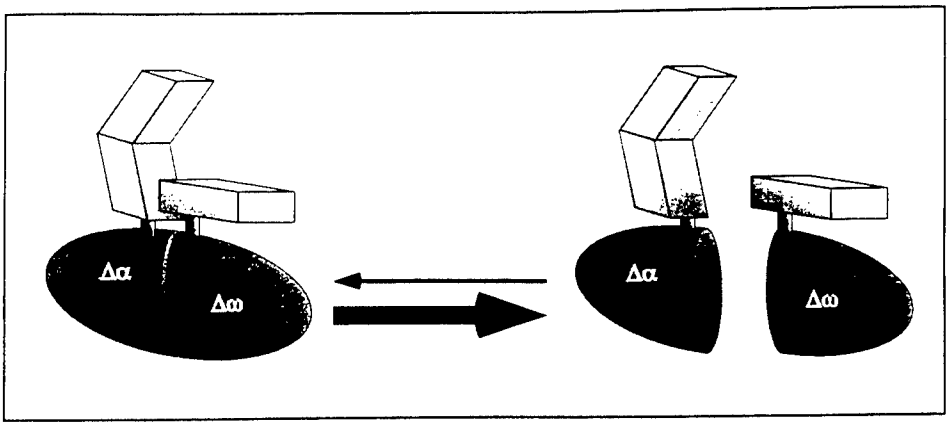

FIG. 1. Schematic of experimental hypothesis. (A) When the $\Delta\alpha$ and $\Delta\omega$ β-Gal mutants are fused to proteins that heterodimerize, their association in the active enzyme conformation is favored. (B) When the two proteins fused to $\Delta\alpha$ and $\Delta\omega$ cannot interact to form a complex, the formation of active β-Gal is not favored.

other more or less efficiently, depending on the nature of the mutations.[7] Our results provide the first demonstration that the same holds true in mammalian cells.[8] Moreover, by using inefficiently complementing mutants to recreate an active β-Gal enzyme, our method allows the interaction of linked protein sequences to be assessed. Thus the β-Gal activity monitors,

[7] M. Villarejo, P. J. Zamenhof, and I. Zabin, *J. Biol. Chem.* **247,** 2212 (1972).
[8] W. A. Mohler and H. M. Blau, *Proc. Natl. Acad. Sci. U.S.A.* **93,** 12423 (1996).

or serves as a readout, for the physical interaction between the non-β-Gal protein components.[9]

There are several significant advantages of the β-Gal complementation method.[10] First, quantitation is possible, as the enzyme that provides the readout of the assay is covalently linked to the interacting proteins. Thus, the number of complemented β-Gal molecules present in the cell correlates with the number of complexes formed by the test proteins, providing a means of quantifying the interaction. Second, the signal is generated directly in the subcellular compartment where the interaction takes place.[9] Finally, the sensitivity of the assay is such that interactions of trace amounts of proteins can be assayed, avoiding perturbations of cellular physiology due to overexpression.[10a] Using this system, we have been able to monitor ligand-induced interactions between two cytoplasmic proteins (FKBP12 and the FRB domain of FRAP),[9] between a cytoplasmic protein and a transmembrane receptor (FADD and FAS), and the formation of homodimers (EGF receptor[10a]) and higher order complexes (FAS) composed of transmembrane proteins.

Development of Mammalian β-Galactosidase Complementation: Historical Perspective

The derivation of a mammalian β-Gal complementation system arose from our interest in muscle cell differentiation. To better understand signaling from cell surface molecules, substrates, or growth factors that have been implicated in the fusion of myoblasts to form multinucleate syncytial myotubes, we sought a rapid, sensitive, highly reproducible biochemical assay to supplant the traditional microscopic scoring of the percentage of nuclei contained inside and outside syncytia. The classic bacterial genetic phenomenon of intracistronic complementation of the *lacZ* gene proved ideal for this purpose.[4] In *E. coli*, deletions of either the N or C terminus of β-Gal produce enzyme that is inactive, but can be complemented by coexpression of an inactive mutant defective in the other terminus. Although in *E. coli*, restoration of enzyme activity results from formation of a heterooctamer of complementing mutant peptides rather than the tetramer that normally constitutes active β-Gal, in mammalian cells the precise

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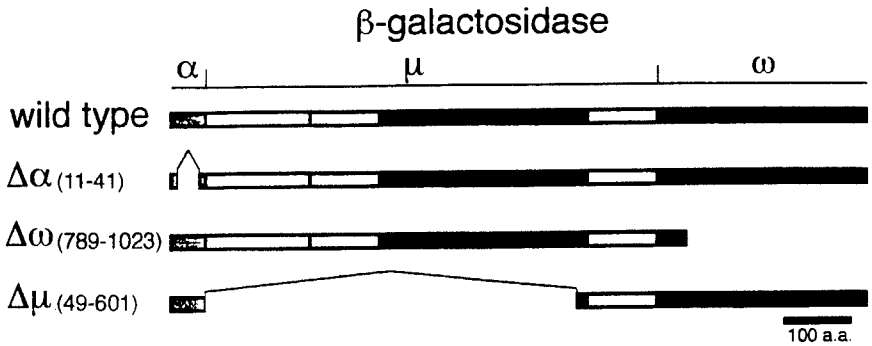

FIG. 3. Schematic representation of the β-Gal mutants. The strongly complementing mutants Δα and Δμ are used to monitor cell fusion.[8] In contrast, the weakly complementing Δα and Δω mutants are also used to monitor protein–protein interactions.[9] [Reproduced from W. A. Mohler and H. M. Blau, *Proc. Natl. Acad. Sci. U.S.A.* **93,** 12423 (1996).]

number of monomers in an active complex remains to be determined.[11–13] On the basis of the published bacterial genetic map, biochemical data, 3D structure (Fig. 2; see color insert), and the existence of convenient restriction sites, we constructed vectors expressing more than 20 deleted versions of *E. coli* β-Gal, including Δα (N-terminal deletion), Δω (C-terminal deletion), and Δμ (a large central deletion; Fig. 3).[6–8,11,14] When NIH 3T3 cells were sequentially infected with pairs of potentially complementing constructs and assayed histochemically for β-Gal activity, certain mutant pairs complemented more efficiently than others. The most strongly complementing pair, Δα and Δμ, was used to develop a fusion assay in which populations of myoblasts independently infected with virus expressing either β-Gal mutant Δα or Δμ were mixed in equal proportions and plated in coculture.[8] After different periods of time in differentiation medium [Dulbecco's modified Eagle's medium (DMEM) plus 5% (v/v) equine serum, changed daily], the cells were assayed for β-Gal activity using a sensitive chemiluminescent substrate (see Chemiluminescence Assay, below). The kinetics of myoblast fusion as assayed by β-Gal activity correlated extremely well with the classically used, labor-intensive microscopic analysis of cell fusion.[15]

The β-Gal complementation assay has since been used to investigate

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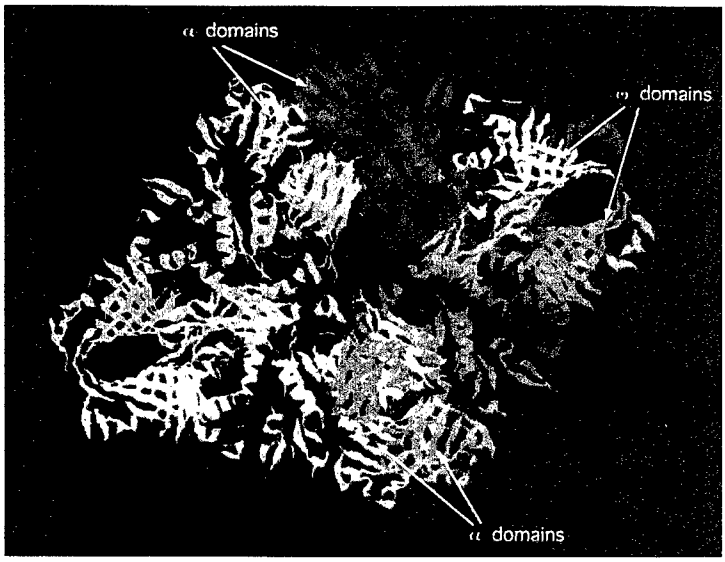

FIG. 2

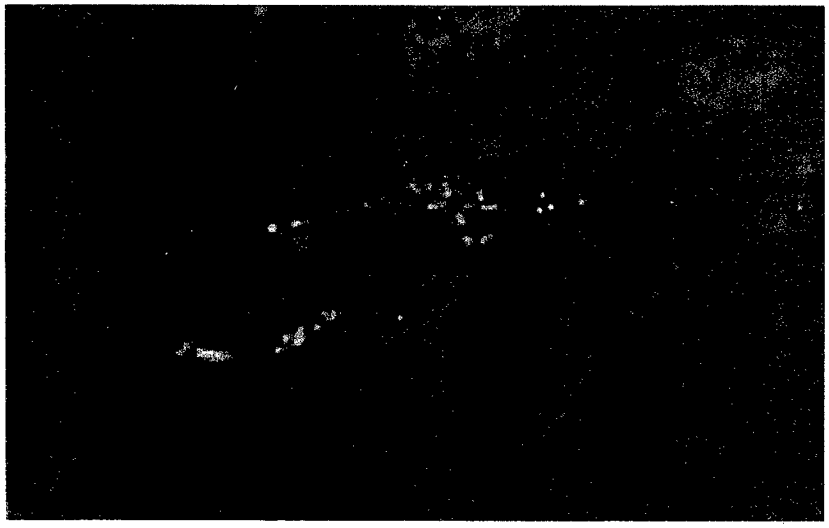

FIG. 4

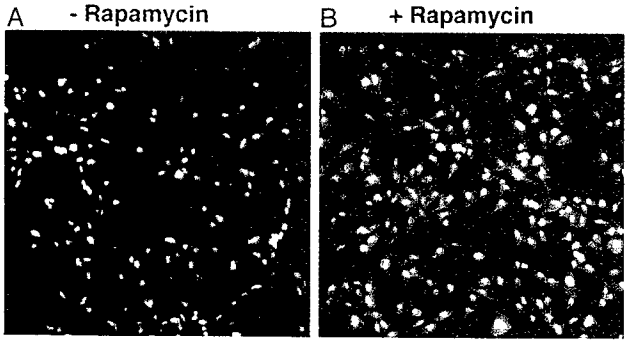

FIG. 7

FIG. 2. Three-dimensional structure of β-Gal in its active tetrameric form. Each monomer is represented in a different color (green, light blue, cyan, and blue). Two α domains are in red and two are in yellow. The two ω domains on the right-hand side of the molecule are represented in gold and orange, respectively. Notice the extensive intramolecular contacts of the α and ω domains with the central portion of each monomer.

FIG. 4. Simultaneous detection of β-Gal activity and multiple other markers. Complemented β-Gal activity could not be visualized in conjunction with immunofluorescence until the development of Fluor X-Gal. Using Fluor X-Gal as the β-Gal substrate (green), nuclei were labeled with DAPI (blue), and the membrane marker NCAM was detected with rat anti-NCAM antibodies followed by biotinylated goat anti-rat secondary antibodies and Cy5-labeled streptavidin. [Reproduced from C. A. Charlton, W. A. Mohler, G. L. Radice, R. O. Hynes, and H. M. Blau, *J. Cell Biol.* **138**, 331 (1996).]

FIG. 7. Induction by rapamycin of β-Gal activity in cells expressing FKBP12–Δω and FRAP–Δα. Cells were maintained overnight either in the absence (A) or in the presence (B) of rapamycin (10 ng/ml). β-Gal activity was visualized by fluorescence histochemistry with Fluor X-Gal as a substrate. Nuclei were stained with Hoechst 33342 (blue) and Fluor X-Gal was viewed with a rhodamine filter (red). [Reproduced from F. Rossi, C. A. Charlton, and H. M. Blau, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 8405 (1997).]

the effect on fusion of various factors and cell adhesion molecules purported to function in myogenesis. The molecules tested to date include α_4-integrin, N-cadherin, and neural cell adhesion molecule (NCAM), all of which had been implicated in myogenic fusion in previous studies.[16-18] The fusion potential of myoblasts that were rendered homozygous null for these adhesion molecules was compared with wild-type cells using β-Gal complementation. Lack of each of the three cell surface adhesion molecules did not have an effect on myoblast fusion in this rigorous assay, suggesting that if they are involved at all, they are not essential.[15,19] This assay should prove generally useful for monitoring fusion in other cell types such as osteoclasts, trophoblasts, and mitotically inactive syncytia found in tumors.

To examine β-Gal activity simultaneously with expression of different cell surface molecules at the single-cell level, we developed a new histochemical assay for β-Gal (see protocol, below). This was necessary because a drawback of the 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) substrate typically used to assay β-Gal activity is that it quenches fluorescence of fluorophores. On the other hand, fluorogenic substrates for β-Gal that are used for live cell sorting [e.g., fluorescein di-β-D-galactopyranoside (FdG)] diffuse readily from the site of enzyme activity.[20] We found that the combination of an azo dye, Fast Red Violet LB, with either X-Gal or with 5-bromo-6-chloro-3-indolyl-β-D-galactopyranoside (5-6 X-Gal) constitutes a substrate (Fluor X-Gal) that is a more sensitive measure of β-Gal activity than the X-Gal substrate alone.[8] The Fluor X-Gal product can be visualized simultaneously with other fluorescent markers (Fig. 4; see color insert), thus allowing for study of the spatial and temporal expression of different molecules in relationship to fusion.[15] This sensitive histochemical assay may also be applicable to monitoring the expression and localization of interacting proteins fused to complementing β-Gal mutants.

β-Galactosidase Complementation Analysis of Protein Interactions

We hypothesized that the less efficient pairs of complementing β-Gal mutants could be used to monitor physical interactions between proteins. This would require that the mutants not drive the reaction. To test this

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hypothesis, we took advantage of the well-characterized interaction between the cyclophilin FKBP12 and the FKBP12–rapamycin-binding domain (FRB domain) of the FKBP12–rapamycin-associated protein (FRAP).[21,22] The interaction between FKBP12 and FRAP is particularly well suited for testing a novel methodology, as it takes place only in the presence of rapamycin, a small cell-permeable drug commonly used as an immunosuppressant. The rapamycin molecule is trapped between the two polypeptides within the FKBP12–rapamycin–FRAP complex and supplies most of the contact interface by binding tightly to both partners.[23] Consequently, FKBP12 and FRAP have no tendency to interact in the absence of rapamycin. Indeed, several laboratories that used this inducible interaction to conditionally target a transcriptional activation domain to a promoter detected no transcription above background in the absence of rapamycin.[24–27] We generated two chimeric proteins in which FKBP12 was fused to $\Delta\omega$ and the FRB domain of FRAP was fused to $\Delta\alpha$ (FKBP12–$\Delta\omega$ and FRAP–$\Delta\alpha$, respectively; Fig. 5). The chimeric cDNAs were inserted into retroviral vectors that also contained genes encoding the selectable markers neomycin or hygromycin resistance downstream of an internal ribosome entry sequence (IRES). After infection of C2F3 cells with retrovirus and selection for resistance to neomycin and hygromycin, the activity of β-Gal was assayed (Fig. 6) in the presence or in the absence of rapamycin (10 ng/ml; higher concentrations of the inducer were toxic to the cells). The cells responded to rapamycin with a detectable increase in β-Gal activity that was first observed after more than 30 min of treatment and reached 30-fold above the background activity assayed in untreated cells after 5 hr of treatment (Fig. 7; see color insert). In addition, the cells responded to concentrations of rapamycin between 0.5 and 10 ng/ml with a linear increase in β-Gal activity, providing evidence that this method allows quantitation of the interaction (Fig. 8). Thus, the response to rapamycin was both time

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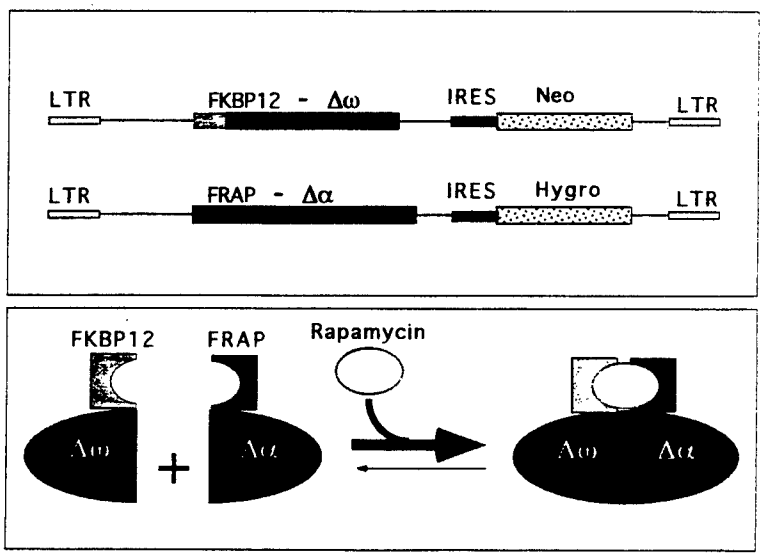

FIG. 5. Use of β-Gal complementation to monitor FKBP12 and FRAP interactions. *Top:* Schematic representation of the FKBP12–Δω–Neo and FRAP–Δα–Hygro retroviral constructs. IRES, Internal ribosome entry sequence; LTR, long terminal repeat. *Bottom:* Rapamycin induces the association of FKBP12 with the FRB domain of FRAP, favoring the reconstitution of functional β-Gal.

and dose dependent.[9] The response was only slightly reduced by treatment of the cells with cytostatic drugs such as mitomycin C. The magnitude of the reduction in β-Gal activity was in good agreement with the expected reduction due to the smaller number of signal-producing cells at given points in time, suggesting that growth-arrested cell populations could be used to facilitate the interpretation of experiments that test protein interactions that affect cell division (Fig. 7).

Application to Membrane Proteins

A major advantage of the β-Gal complementation system for analyzing protein interactions is its ability to detect interactions in the cellular compartment in which they normally occur, in either individual live cells or in mass cultures. The β-Gal complementation assay can be used to detect interactions between two membrane proteins, or between a membrane protein and a cytoplasmic protein. For example, if chimeric proteins consisting of the epidermal growth factor (EGF) receptor linked to two complementing β-Gal proteins are expressed in a single cell, addition of ligand

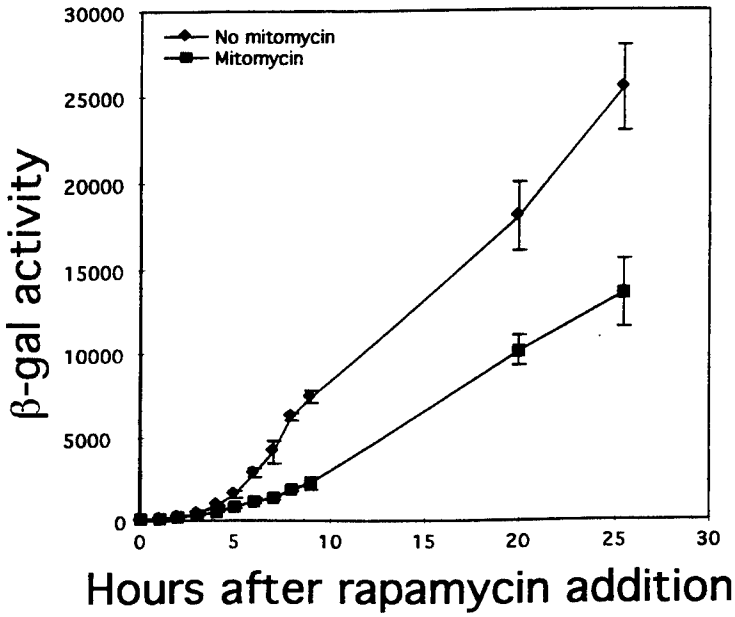

FIG. 6. Kinetics of induction of β-Gal activity on treatment with rapamycin in the presence or absence of the cytostatic drug mitomycin C. C2C12 cells expressing both FKBP12–Δω and FRAP–Δα were either treated with 2 μg of mitomycin overnight or left untreated. Both types of culture were then treated with rapamycin (10 ng/ml). Cells were lysed at different time intervals and the β-Gal activity in the lysates was quantified by chemiluminescence. β-Gal activity is expressed as luminescence counts per second. Each point represents the average of six replicate samples and error bars express standard deviations of the mean.

(EGF or other EGF receptor ligands) results in an increase in β-Gal activity within minutes. β-Gal activity declines after removal of the ligand, and can be induced on addition of fresh ligand. Moreover, an increase in β-Gal activity is prevented by the addition of molecules that inhibit ligand binding.[10a]

This ligand-dependent and ligand-specific β-Gal activity presents several advantages over other methods currently in use or in development: First, it is fast; second, it is direct; third, it occurs *in situ*. Traditionally, dimerization or activation of membrane receptors has been measured by assaying for some downstream event, such as phosphorylation of the receptor or a receptor substrate (in the case of receptor kinases). Such gel electrophoresis-based assays are laborious and time-consuming, and because they permit the analysis of only a few samples at a time, are not adaptable to high-throughput screening technology. Other traditional methods for detecting membrane protein interactions include immunopre-

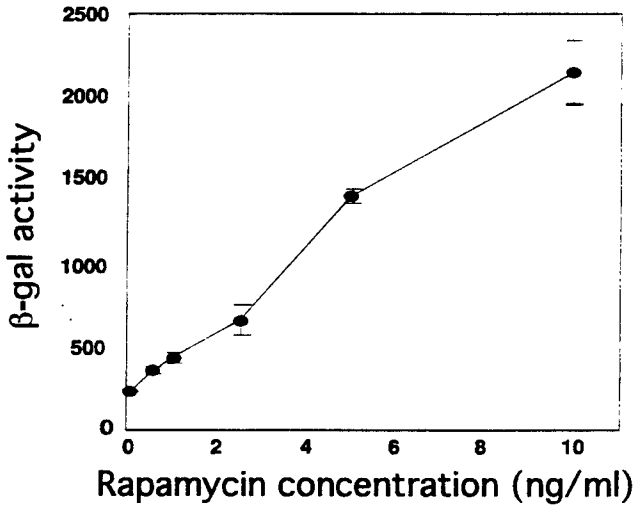

FIG. 8. Dose response to rapamycin of C2C12 cells expressing both FKBP12–$\Delta\omega$ and FRAP–$\Delta\alpha$. Cells were treated for 5 hr with the indicated concentrations of rapamycin, lysed, and β-Gal activity in the lysates was quantified by chemiluminescence. β-Gal activity is expressed as luminescence counts per second. [Reproduced from F. Rossi, C. A. Charlton, and H. M. Blau, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 8405 (1997).]

cipitation, chemical cross-linking followed by immunoprecipitation, or density gradient centrifugation. These methods suffer the same limitations as other gel electrophoresis-based assays, and also detect only a subset of interactions, as some interactions do not survive the disruption of the cell.

Other methods such as fluorescence resonance energy transfer (FRET) assays have been developed to study protein interactions in live mammalian cells.[28] FRET-based assays exist in two forms. In the context of membrane receptors, fluorescent ligands can be added to the cell. This method has been used to analyze ligand binding and receptor dimerization in cells via microscopy.[29] Alternatively, chimeric interacting proteins can be expressed that have a fluorescent molecule, such as variants of green fluorescent protein (GFP), linked to the protein of interest. In this case, FRET analysis can be used to measure the interaction of the fluorescently labeled pro-

[28] S. R. Adams, A. T. Harootunian, Y. J. Buechler, S. S. Taylor, and R. Y. Tsien, *Nature (London)* **349**, 694 (1991).

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teins.[30,31] FRET analysis is limited by spatial constraints and by the sensitivity of detection because there is no signal amplification.

More recently, protein interactions have been monitored with chimeric proteins that complement and reconstitute a functional protein. One such method is the complementation of β-Gal protein as described here. Another is complementation of dihydrofolate reductase (DHFR). Complementation, which restores the ability of DHFR to bind a fluorescent inhibitor, methotrexate, is measured after a prolonged (22-hr) incubation with the inhibitor.[32–34] As with GFP tagging, a disadvantage of DHFR complementation is that it lacks signal amplification, as the assay does not measure enzymatic activity. Whereas one active β-Gal complex can cleave many fluorogenic molecules, each DHFR-bound methotrexate molecule or GFP-tagged protein constitutes a single fluorescent molecule. Thus, expression levels of the protein must exceed a threshold level before an interaction event can be detected. This can be a problem for some proteins, such as the EGF receptor (EGFR), as high levels of receptor expression are known to lead to ligand-independent dimerization.[29] This problem is overcome with the β-Gal system described here, as proteins in low abundance can be detected, allowing ligand-dependent dimerization of the EGF receptor to be readily monitored.[10a] Furthermore, β-Gal substrates can be introduced into live cells within minutes and results obtained rapidly.[20] The 1-day period required for the DHFR assay suggests that transient interactions may be missed. In addition, it remains unclear whether the DHFR technology can be used in quantitative assays in wild-type cells with endogenous, active DHFR.

Of particular importance was the finding that the kinetics of β-Gal activity in response to an inducer varied with the different protein pairs. When the dimerization of chimeric EGFR–β-Gal constructs was induced with EGF, an increase in β-Gal activity was detected with less than 1 min of treatment with EGF, and enzyme activity continued to increase for another 1 to 2 hr.[10a] In contrast, in the case of the rapamycin-dependent FKBP12–FRAP interaction, β-Gal activity was not detectable until after more than 30 min of treatment with the inducer, rapamycin, and continued to increase over the subsequent 12 hr.[9] These kinetics of association fit well

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with those previously reported for rapamycin-binding proteins.[24,27] These findings indicate that the time course of β-Gal activity reflects the characteristics of the non-β-Gal protein interactions being monitored rather than the kinetics of the complementation reaction. The reconstitution of β-Gal activity by intracistronic complementation is thought to require partial refolding of the mutants and subsequent assembly of the tetrameric active enzyme.[6,11,12] In theory, a delay might be expected between the time of formation of the complex containing the test proteins and the development of a detectable β-Gal signal. However, our experiments suggest that such a lag is on the order of minutes and should therefore be negligible for most applications.

Potential Applications and Future Directions

Because the β-Gal complementation system is a rapid, enzymatic assay, it is amenable to high-throughput screening technologies, such as simple multiwell microplate assays that yield results within 1 hr (see Chemiluminescence Assay, below). The most straightforward and immediate application of this method is likely to be the screening of combinatorial chemical libraries in order to identify compounds that can either block or induce a specific interaction. This method is particularly well suited to the monitoring of interactions between membrane proteins such as receptors, or between receptors and cytoplasmic components of a given downstream signaling pathway. For example, cells expressing EGF receptor chimeras can be plated in multiwell plates and used to screen a library of chemical compounds or proteins for molecules that act as receptor agonists, or antagonists. This could be of particular interest for a molecule such as the EGF/erbB receptor family member erbB2, which has been implicated in a number of cancers, particularly breast cancer.[35] Overexpression of the erbB2 receptor leads to constitutive dimerization and activation of the receptor.[36] Molecules that block the dimerization and thus activation of erbB2 may be useful in anticancer therapy. Furthermore, known ligands of EGF receptor family members can easily be tested on cells expressing different combinations of erbB receptors, which could lead to a better understanding of the homo- and heterodimerization events that occur in this receptor family and underlie normal or neoplastic cell growth.[37–39]

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We have found that the β-Gal complementation methodology requires only a low level of expression of the chimeric proteins to generate a detectable signal on interaction. Indeed, the lower the expression, the lower the frequency of nonspecific ligand-independent interactions.[10a] Thus it is possible that in most cases, the levels of expression that can be detected will be physiological and comparable to the expression levels of the endogenous proteins. These characteristics of β-Gal complementation may make it applicable to detailed studies of protein interactions involved in signaling pathways. Furthermore, β-Gal complementation may allow direct monitoring of protein interactions in transgenic animals. Specific chimeric proteins could be constructed and tested *in vitro* for their ability to generate β-Gal activity on interaction. The best chimeras could then be recreated *in vivo* by "knock in" of $\Delta\alpha$ and $\Delta\omega$ in frame with the endogenous genes. Animals carrying both transgenes should develop β-Gal activity only in those cells in which the endogenous proteins are expressed and actually interact. Histochemical detection of β-Gal in these animals models should allow the mapping of specific protein interactions both during development and in disease states. Such studies would allow an advance from gene expression to gene function *in vivo*.

Mammalian Two-Hybrid System

Another exciting potential application of β-Gal complementation stems from the possibility of screening a library of cDNAs fused to β-Gal mutants in order to identify genes encoding novel protein interaction partners in mammalian cells. Although the yeast two-hybrid system and its modifications have enabled researchers to screen cDNA libraries for gene products interacting with a known protein, leading to a great increase in the number of known interaction pairs, the β-Gal complementation system may have distinct advantages. In the simplest and most common embodiment of the yeast two-hybrid system, the interaction between a DNA-bound protein (bait) and a polypeptide derived from a cDNA expression library targets a potent transcriptional activation domain to a synthetic promoter and leads to transcription of a detectable marker gene. This interaction-dependent activation of transcription has the advantage of greatly amplifying the signal. Indeed, the yeast two-hybrid system can easily detect low-affinity interactions.[3] However, it also presents disadvantages: for transcription to be activated, the interaction must take place in the nucleus; interactions that take place only in other cellular compartments, such as membranes, will not be detected by this method. Furthermore, attempts to adapt this system for use in mammalian cells have met with limited success.

The adaptation of β-Gal complementation to two-hybrid screens offers

several advantages over the yeast system. In theory, a chimeric library obtained by fusing random cDNAs to one of the β-Gal mutants could be constructed. This library could then be screened for gene products that interact with a given "bait" protein fused to the complementary β-Gal mutant. Such a screen would be performed in cultured mammalian cells, allowing the detection of interactions that need to be facilitated by an endogenous protein, or that take place only in a specific cellular compartment. The screen would be facilitated by the availability of flow cytometry-based techniques that allow the isolation of β-Gal-positive cells without compromising their viability[20] (Fig. 9). Furthermore, because the readout of the assay would not be dependent on the nuclear localization of the interacting protein partners, this method could be used to isolate partners of membrane-linked proteins. Such a mammalian two-hybrid screen should lead to the identification of signal transduction components in particular cell contexts (e.g., normal vs neoplastic) and, ultimately, new drug targets.

Practical Issues in Using β-Galactosidase Complementation to Monitor Protein–Protein Interactions

To monitor the interaction between two proteins, each of the proteins is fused to one of a pair of β-Gal mutants (Fig. 1), and the two fusion proteins are expressed at low levels in mammalian cells. The induced interaction between the two test proteins will then facilitate the complementation reaction and result in an increase in β-Gal activity. For this type of application, we have developed a specific pair of β-Gal deletion mutants ($\Delta\alpha$ and $\Delta\omega$) that display a low level of spontaneous complementation

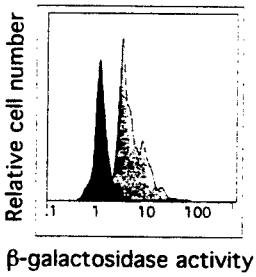

FIG. 9. Fluorescence-activated cell sorter (FACS) analysis of rapamycin-induced β-Gal complementation. The induced and uninduced populations yield essentially nonoverlapping peaks. The vertical axis represents relative cell number, and the horizontal axis depicts intensity of fluorescence on a logarithmic scale. The peaks on the left represent the untreated samples and those on the right represent samples treated with rapamycin (10 ng/ml).

when coexpressed in mammalian cells.[8] Nevertheless, to avoid background β-Gal activity that could mask the interaction-dependent complementation, it is critical that the expression of the chimeric proteins be kept relatively low.[9] This should be an advantage in most cases: Low-level expression is less likely to perturb the balance of proteins in the cell and lead to nonphysiological results than the high levels of expression required by other systems that do not provide any signal amplification. To control the expression levels of the chimeric proteins, we favor the use of IRES-containing retroviral vectors expressing the chimeric protein and a selectable marker from the same bicistronic messenger RNA.[40] Unlike the use of plasmid transfection, which usually results in multiple copies of the vector in the genome of the selected stable clones, infection with retroviral vectors permits the introduction of a single copy of the expression cassette in the target cells, thereby reducing the chances of overexpression. Furthermore, in our experience, bicistronic mRNAs tend to yield lower protein expression levels compared with shorter mRNAs containing a single coding sequence.

A variety of assays are available to detect β-Gal activity, reflecting its extensive use as a marker over three decades. We have successfully detected "complemented" β-Gal in mammalian cells by histochemistry with both the traditional chromogenic substrate X-Gal and a novel, more sensitive fluorogenic substrate (see Histochemical Assays, below). These detection methods, however, are not quantitative and require fixation of the cells. Sensitive quantitative detection of complemented β-Gal can be achieved by assaying cell lysates with commercially available chemiluminescence substrates [e.g., the Gal-Screen kit from Tropix (Bedford, MA); see Chemiluminescence Assays, below]. Chemiluminescence assays in multiwell microplates permit a rapid analysis of the effect of a variety of pharmacological and biological agents on the protein interactions of interest under a range of conditions. Quantitative analysis is also possible in live cells by using the fluorogenic substrate fluorescein di-β-D-galactopyranoside (FdG) followed by detection by flow cytometry (see Flow Cytometry Assay, below, and Fig. 9).[20] FdG can be introduced into live cells by hypotonic loading and the fluorescent cleavage product of the reaction catalyzed by β-Gal (free fluorescein) will remain within the cell as it is unable to cross the plasma membrane. The fluorescence-activated cell sorting (FACS) assay has the further advantage of allowing recovery of live cells in which a given interaction has taken place.

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Molecular Basis of Interaction-Induced β-Galactosidase Complementation

How does forcing the interaction between inefficiently complementing β-Gal mutants increase their ability to recreate an active enzyme? For a hypothesis to be generated, it is necessary first to consider what limits the ability of the Δα and Δω mutants to complement each other spontaneously. For the complementation reaction to take place, one of the two mutants must be able to provide in *trans* the critical domain that the other mutant lacks. For example, in α-complementation in *E. coli*, a peptide containing the α portion of β-Gal "donates" this domain to a β-Gal mutant that harbors a deletion in the α region, a process that requires the establishment of noncovalent interactions between the two proteins.[11,12] Consequently, a critical requirement for the re-creation of enzymatic activity is that the relevant domain is available to be "donated." An analysis of the crystal structure of wild-type β-Gal clearly indicates that both the α domain and the ω domain that are absent from the Δα and Δω mutants are involved in extensive intramolecular interactions with the central portion of the molecule[14] (μ domain; Fig. 2). As both Δα and Δω contain a complete μ domain, it is likely that in these mutants the intact ω and α domains (either of which could in theory be "donated" to the complementary mutant) are sequestered in such intramolecular interactions. Thus, they would not be readily available to establish the intermolecular interactions with the μ domain of the complementary mutant required to reconstitute a functional monomer. Support for this hypothesis comes from the finding that in inefficient α donors, in contrast to efficient α donors, the α domain is not accessible to proteolytic enzymes, suggesting that it is probably masked by intramolecular interactions.[7,13] When the two β-Gal mutants are forced to come in close contact by fusion to interacting proteins their effective local concentration increases greatly, the intermolecular interaction is more likely to occur, and consequently the rate of complementation is increased, generating a detectable signal. Future analysis of crystal structures of the complementing mutants will certainly be instructive.

Troubleshooting

In theory, the use of the β-Gal complementation method for detecting protein–protein interactions may be subject to steric constraint. On interaction of the test proteins, Δα and Δω may need to associate with a certain optimal orientation in order to generate detectable enzymatic activity. This is likely to depend on the orientation of the test proteins within the complex they form. For example, two chimeric proteins fused to the same end of

the β-Gal mutants may not lead to efficient complementation if they interact in a head-to-tail fashion, as the β-Gal mutants may project out of the complex in opposite directions and thus never contact each other. The same could happen if the test proteins are too bulky. It is unlikely, however, that these theoretical limitations will preclude the analysis of specific interactions. Given two known polypeptides, it should be possible to generate a range of chimeric proteins *in vitro* and select those that display the best characteristics for later use in, for example, high-throughput screens or transgenic animals. This limitation, however, may preclude the detection of a subset of cDNAs in a library screen, because the cDNA–β-Gal fusion points are necessarily generated at random and cannot be predicted or individually optimized. Studies to optimize interactions and overcome these potential problems are underway, for example, the inclusion of linkers of different lengths between the test protein and β-Gal.

Another limitation affecting this method stems from the fact that available substrates easily localize β-Gal activity to individual cells within a population, but are not efficient in localizing the enzyme at the subcellular level. Whereas nuclear β-Gal can be distinguished from cytoplasmic β-Gal, a more refined localization of the enzyme is prevented by the propensity of the existing substrates to diffuse. Such a problem underscores the need to develop less diffusible substrates, and work is underway to address this need.

Concluding Remarks

Clearly, the application of intracistronic β-Gal complementation to monitoring protein–protein interactions in live cells has tremendous potential, but is still in its infancy. Improvements will certainly be forthcoming as well as increased insights into the underlying molecular mechanisms. Nevertheless, the β-Gal system already allows an investigation of fundamental questions regarding protein interactions involved in cell signaling such as membrane receptor dimerization. High-throughput screens for antagonists and agonists of specific protein interactions can be envisioned. In addition, the development of a "mammalian two-hybrid" system may soon become a reality.

Methods

Cloning, Retrovirus Production, and Infection

To produce the infectious viral particles, the Phoenix-E ecotrophic packaging cell line (a kind gift of G. Nolan, Stanford University) is transiently transfected with the plasmids containing the appropriate proviral genome.

Cells are plated in 60-mm plates the day before transfection at 3×10^6 cells/plate, and transfected with Fugene 6 (Roche, Indianapolis, IN) according to the manufacturer instructions. With this cell line, we obtain optimal transfection with 6 μl of Fugene and 2 μg of plasmid DNA per plate. Although other methods, including the traditional calcium phosphate transfection protocols, can sometimes result in transfection of a higher percentage of the cells, we consistently obtain higher viral titers with Fugene 6, possibly because of the absence of toxic side effects that may affect viral production. Tissue culture supernatant containing the infectious particles is harvested between 36 and 72 hr after transfection and used to infect C2F3 myoblasts plated in 60-mm plates. In some cases, depending on the efficiency of transfection and virus production, we dilute the supernatants 1:10 in fresh medium to limit the number of vector copies introduced in each target cell. Infections are carried out either sequentially or simultaneously by replacing the C2F3 medium with viral supernatant filtered through a 0.45-μm pore size filter. Polybrene is added at a final concentration of 8 μg/ml and the plates containing the target cells are centrifuged in a tabletop Beckman (Fullerton, CA) GS-6 centrifuge equipped with microplate platforms at 2500 rpm for 30 min. The centrifugation step leads to an increase in infection efficiency, but it can be omitted as high multiplicity of infection is not desirable for most applications of this method. Selection with hygromycin and/or neomycin (0.8 mg/ml of each drug) is started 24 hr after infection.

Flow Cytometry Assay

To date, a selection strategy that imparts a growth advantage specifically to cells that express β-Gal is not available. However, β-Gal can be detected in live cells with fluorescent substrates, and fluorescent cells can be efficiently separated from mixed populations rapidly and efficiently by flow cytometry without affecting their viability. This assay is relatively simple and can be performed in less than half an hour when the number of samples to be analyzed is limited. Furthermore, it is quantitative and we use it routinely to monitor the kinetics of a given interaction in response to specific signals. The traditional substrate used for this assay, fluorescein di-β-D-galactopyranoside (FdG), is not cell permeable but it can be introduced into the cells by hypotonic shock.[20] Cleavage by β-Gal results in the production of free fluorescein, which is also unable to cross the plasma membrane and is trapped inside the β-Gal-positive cells. The cells to be analyzed are trypsinized, resuspended in phosphate-buffered saline (PBS) containing 5% (v/v) fetal bovine serum (PBS–FBS), and pelleted in 5-ml polystyrene round-bottom tubes (Falcon 2058; Becton Dickinson Labware, Lincoln

Park, NJ). The cells are then resuspended in 100 μl of PBS–FBS and an equal volume of doubly distilled water containing the substrate at a concentration of 1 m*M* is added. After 3 min at room temperature, the hypotonic conditions are quenched by adding 10 volumes of ice-cold PBS–FBS containing propidium iodine (PI, 1 μg/ml). PI is a red fluorescent compound that is actively excluded from living cells but accumulates in dead cells, allowing their exclusion from the analysis or the sorting. After quenching, the cells are pelleted again, resuspended in approximately 200 μl of ice-cold PBS–FBS, and analyzed on a Becton Dickinson (San Jose, CA) FACScan or sorted on a Becton Dickinson FACStar flow cytometer. In most clonal populations, the cells respond to signals inducing the test interaction with an homogenous increase in β-Gal activity, and the mean fluorescence in each sample can be used as a reliable measure of the interaction. In polyclonal populations, however, we have often noticed that a subset of the cells does not respond to the inducing signal, and the mean fluorescence is less useful. However, the main advantage of the FACS-based assay is that it provides a simple means of selecting for cells in which a given interaction takes place, opening the path to screening cDNA libraries for novel interaction partners of a given "bait" protein in mammalian cells. This rapid method of selection is in our opinion superior to growth-based selection protocols, as many interactions are likely to be transient under physiological conditions and could possibly affect the growth characteristics of the cells.

Histochemical Assays

X-Gal Histochemistry. Chromogenic detection of β-Gal activity can be performed on cells cultured in plastic tissue culture dishes or on glass coverslips. Cells are fixed for 4 min in cold (4°) 4% (w/v) paraformaldehyde in PBS and rinsed for 5 min, two times. A stock solution of X-Gal [Sigma (St. Louis, MO); 40 mg/ml in dimethylformamide, stored at −20°, protected from light] is diluted to a final concentration 1 mg/ml in 5 m*M* $K_3Fe(CN)_6$, 5 m*M* $K_4Fe(CN)_6$, 2 m*M* $MgCl_2$ in PBS, applied to cells, incubated at 37° overnight (shorter times are sufficient for high levels of β-Gal activity), and examined microscopically for blue cells.

Fluor X-Gal Histochemistry. Muscle cells to be labeled for fluorescent histochemical detection of β-Gal activity are cultured on sterile collagen-coated glass coverslips (Becton Dickinson), fixed in 4% (w/v) paraformaldehyde in PBS, and rinsed twice with PBS (in which they can be stored at 4° until staining is performed). If cells are to be labeled with antibody as well as Fluor X-Gal, cells are blocked for 30 min in PBS + 10% (v/v) equine serum, incubated for 2 hr in primary antibody, rinsed four times (10 min

each) in blocking buffer, incubated for 1 hr in biotinylated secondary antibody, washed again four times in blocking buffer, incubated for 1 hr in Cy5-labeled streptavidin (diluted 1:100; Amersham, Arlington Heights, IL), and then washed two times in blocking buffer and two times in PBS. All immunolabeling steps are performed at 4°. β-Gal substrate is prepared by diluting a stock solution of Fast Red Violet LB (Sigma; stock is 50 mg/ml in dimethylformamide, stored at −20°, not totally dissolved at this concentration) to a final concentration of 100 μg/ml and a stock solution of 5-6 X-Gal [5-bromo-6-chloro-3-indolyl-β-D-galactopyranoside Fluka (Ronkonkoma, NY); stock at 50 mg/ml in dimethylformamide, stored at −20°, will change from pale blue to yellow after exposure to light, but this does not appear to affect activity] mixed to a final concentration of up to 25 μg/ml in PBS (decrease the concentration if β-Gal activity is strong). A 0.45-μm pore size syringe filter is used to remove any precipitate. The mixture of Fast Red Violet LB and 5-6 X-Gal is added to fixed cells and incubated 60 to 90 min at 37°, and then rinsed in 25 ml of PBS for 30 min at room temperature. Nuclei may be stained by diluting 4′,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; Sigma) in PBS to a final concentration of 100 ng/ml, incubating for 10 min at room temperature, and rinsing twice for 5 min. Coverslips are mounted in PBS (no glycerol-based antifade solution) and sealed with nail polish. Fluor X-Gal staining can be viewed with either fluorescein isothiocyanate (FITC) or rhodamine (TRITC) filter sets of an epifluorescence microscope (signal-to-noise peak is 560 nm for weak signals; the rhodamine filter set has a 560-nm bandpass emission filter). The FITC channel gives a better signal-to-background ratio for weak signals, but strong signals appear to be quenched. Therefore, Fluor X-Gal stain is best viewed with TRITC filters (Figs. 4 and 6).

Chemiluminescence Assay

Quantitative assays for β-Gal activity are carried out in 96-well plates with a commercial chemiluminescent assay system with prolonged (glow) emission. Although the FACS-based assay is important for characterization at the single-cell level of live cells expressing β-Gal chimeric proteins, it is difficult to prepare more than about 150 samples at a time for FACS analysis. The chemiluminescent assay described here permits the analysis of thousands of samples in one experiment. Numerical results can be obtained 1 hr after the assay is begun.

Subconfluent tissue culture cells are trypsinized, counted in a cell counter (Z1; Coulter, Hialeah, FL), and replated in white 96-well plates at 10,000 cells per well (for C2F3 mouse myoblasts) in a volume of 100 μl the day before the assay. Plates are maintained in an appropriate medium

and incubator conditions [DMEM with 20% (v/v) FBS at 37° in 10% CO_2 for C2F3 cells] up to the addition of the chemiluminescent reagent. We have tested white 96-well plates with white opaque bottoms and clear bottoms (which permit viewing cells on a microscope). Opaque plates yield higher absolute luminescence values; however, the signal-to-background ratio in the two types of plates are similar. In clear bottom plates, luminescence from a strongly luminescent sample can be measured in an adjoining empty or weakly luminescent well. Such cross-talk is prevented in the fully opaque plates.

Cells are treated with agents that stimulate protein interactions of chimeric β-Gal proteins as desired. For example, cells are treated for minutes or hours with a growth factor that induces dimerization of a chimeric growth factor receptor–β-Gal protein. Normally, addition of agents is accomplished by replacing the medium in the well with fresh medium containing the agent, maintaining a volume of 100 μl per well. The medium in untreated wells is also replaced at the same time, as background luminescence (luminescence measured in wells containing only medium and the chemiluminescence reagents) increases the longer the medium (DMEM in our case) is left in the well. Wells are grouped into triplicate or quadruplicate samples for treatment. If wells are to be treated for different times, the start of treatment must vary so that all treatments in a given plate end at the same time, or the different time points must be on separate plates. In the latter case, controls must be present on each plate to control for any variations in the chemiluminescent assay.

At the end of the treatment time, the Tropix Gal-Screen chemiluminescent reagent is added to the plates. Tropix Gal-Screen (GSY200 or GSY1000) consists of two components. The Gal-Screen substrate (1,2-dioxetane compound) is diluted 1:25 immediately before use into Gal-Screen buffer B equilibrated to room temperature. Gal-Screen (100 μl) is added to each well without removing the medium. The plate is then incubated at 26 to 28° for 45 min to 1 hr, and is then read on a plate reader, measuring each well for 1 sec. All readings, especially if multiple plates are used, are made in the period of time in which the chemiluminescence has plateaued, which is typically in the 45-min to 2-hr range.

The following plate readers offer comparable sensitivity: Tropix TR717, EG&G Wallac Berthold (Bad Wildbad, Germany), LB96V, Wallac/EG&G MicroBeta Plus (current version is MicroBeta Trilux). All these machines have optional injectors. On the MicroBeta, addition of an injector to the machine reduces the sensitivity of the instrument. On the TR717 and LB96V, the presence of an injector does not affect the sensitivity of the instrument. The MicroBeta has the advantage of being able to count multiple plates unattended. However, it takes 5 to 10 min to count one

plate whereas the TR717 and LB96V, which hold only one plate at a time, require about 2 min to count a plate. The data are then analyzed in a Microsoft Excel spreadsheet. Replicate samples are averaged together, and the luminescence value of wells containing only medium, or containing cells lacking the β-Gal constructs, is subtracted from the measured values.

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