Molecular Imaging of Protein-Protein Interactions: Controlled Expression of p53 and Large T-Antigen Fusion Proteins in Vivo

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ABSTRACT

Protein-protein interactions control essential steps in signal transduction pathways and other intracellular processes, and assembly of protein complexes modulates and responds to the regulatory events that exist in living animals. We have used microPET and fluorescence imaging to detect interactions between p53 tumor suppressor and large T antigen (TAg) of SV40 virus in a tetracycline-inducible two-hybrid system. To additionally validate this molecular imaging technique, we investigated whether expression of the reporter gene, comprised of a mutant thymidine kinase from herpes simplex virus 1 fused to green fluorescent protein could quantify relative differences in amounts of interacting hybrid proteins. In HeLa cells stably transfected with the reporter gene and interacting (p53-TAg) or noninteracting (p53 and polyoma virus coat protein) pairs of proteins, treatment with doxycycline produced time- and dose-dependent increases in expression of hybrid proteins. Proportional increases in amounts of reporter gene were produced only in cells expressing p53 and TAg. In mice bearing xenografts of these stably transfected HeLa cells, amounts of hybrid proteins were regulated with doxycycline. Both microPET imaging and biodistribution studies showed time- and dose-dependent increases in accumulation of the reporter substrate 9-[(18F)-fluoro-3-hydroxymethylbutyl]guanine only in p53-TAg tumors. Fluorescence microscopy of excised tumors also showed corresponding changes in expression of the fusion reporter gene in response to binding of p53 and TAg. These data demonstrate that the imaging two-hybrid system responds in a proportional fashion to increasing amounts of interacting proteins in vivo.

INTRODUCTION

Protein-protein interactions control most cellular processes, including signal transduction, cell cycle progression, and metabolic pathways. Molecular mechanisms regulating protein interactions have been defined primarily through studies in vitro and in cultured cells. Investigations in these model systems already have identified many different interconnecting circuits of protein complexes, and efforts are ongoing to develop comprehensive maps of protein-protein interactions (1). Because assembly and dissociation of protein complexes are regulated by and mediate signaling pathways, the tissue and physiological environment closely control protein-protein interactions in vivo (2). Therefore, important insights into regulation and biological effects of protein-protein interactions likely will be obtained by studying protein interactions in the setting of an intact, living organism.

A variety of different techniques have been used to detect protein interactions in vitro and in cultured cells, including two-hybrid assays (3), protein-fragment complementation (4, 5), split-ubiquitin (6), and mass spectrometry (7). Of these methods, two-hybrid assays have been used most extensively to characterize interactions between known binding partners and to identify new pairs of interacting proteins (8). Two-hybrid systems exploit the modular nature of transcription factors, many of which can be separated into discrete DNA-binding and ADs (3). Proteins of interest are expressed as fusions with either a DNA BD or AD, creating hybrid proteins. If the hybrid proteins bind to each other as a result of interaction between the proteins of interest, then the separate BD and AD of the transcription factor are brought together within the cell nucleus to drive expression of a reporter gene. In the absence of specific interaction between the hybrid proteins, the reporter gene is not expressed, because the BD and AD do not associate independently. Two-hybrid assays can detect transient and/or unstable interactions between proteins, and the technique is reported to be independent of expression of endogenous proteins (1). Although two-hybrid assays initially were developed in yeast, the technique also has been adapted for use in bacteria and cultured mammalian cells.

Two-hybrid assays were developed recently to detect protein-protein interactions in living mice with noninvasive molecular imaging techniques (9, 10). Specifically, we described a tetracycline-inducible system for imaging interactions between p53 tumor suppressor and large TAg from SV40 virus, using a reporter comprised of a mutant HSV1-TK fused to GFP. To produce hybrid proteins, we fused p53 with the Gal4-BD from Saccharomyces cerevisiae and TAg with the VP16-AD from HSV1, respectively. HeLa cells were transfected stably with the reporter gene, and tetracycline-regulated hybrid p53 and TAg, and these cells were used to establish tumor xenografts in mice. After inducing hybrid p53 and TAg with doxycycline, interactions between these two proteins activated expression of the reporter gene in living mice, which was detected with microPET imaging and fluorescence. In the absence of interacting proteins, background levels of reporter were observed by imaging and biodistribution studies, showing specificity of the two-hybrid system in vivo.

Whereas our initial research demonstrated that a specific protein-protein interaction could be imaged in living animals, these data did not determine whether relative differences in amounts of interacting proteins and expression of reporter gene could be quantified with the imaging two-hybrid system. Furthermore, the time interval between induction of interacting proteins and detection of reporter activity in vivo was not established. Characterizing these properties of the imaging system is essential to determining the sensitivity of the system and range of biological hypotheses that can be investigated in vivo. In the present study, we define these quantitative and kinetic parameters in cultured cells and living mice. Our data show that microPET imaging can detect and quantify interactions between differing amounts of p53 and TAg as early as 12 h after inducing expression of hybrid proteins in vivo. These results additionally validate two-hybrid imaging as a biotechnology for analyzing protein-protein interactions in the context of an intact animal.

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3 The abbreviations used are: BD, binding domain; AD, activation domain; TAg, T antigen; TK, thymidine kinase; HSV, herpes simplex virus; GFP, green fluorescent protein; Gal4-BD, Gal4-DNA binding domain; VP16-AD, VP16 activation domain; PCV, penciclovir; 18F-FHBG, 9-[(18F)-fluoro-3-hydroxymethylbutyl]guanine; ROI, region-of-interest; SUV, standard uptake value; PET, positron emission tomography; CP, coat protein.
MATERIALS AND METHODS

Plasmids. Constructs for Gal4-mNLS-39Tk-EGFP (denoted mTK-EGFP in this report), pRES-r-tTA2, pBlGAL4-BD-p53/VP16-TAg, and pBlGAL4-BD-p53/VP16-CP have been described previously (10).

Cell Lines. HeLa cells stably transfected with mTK-EGFP, pRES-r-tTA2, pBlGAL4-BD-p53/VP16-TAg, and pBlGAL4-BD-p53/VP16-CP were isolated and maintained as described (10). Two subclones of Gal4-BD-p53/VP16-TAg cells (designated as TAg 453 and TAg 478 cells) with differing levels of reporter enzyme induction were isolated from the originally described line of positive-interacting HeLa cells with continued selection in 2.5 μg/ml blasticidin, 500 μg/ml G418, and 50 μg/ml hygromycin. All of the selection drugs were removed from growth medium one day before addition of doxycycline for cell culture experiments or injection into nude mice for tumor xenografts.

Radiochemicals. 8-[^3]H]PCV (14.9 Ci/μmol) was obtained from Moravek Biochemicals (Brea, CA). 18 F-FHBG (∼300 Ci/ml) was synthesized in the Washington University Molecular Imaging Center Chemistry Core as described (11, 12) using a modified procedure of the synthesis reported by Alaeddin and Conti (13).

In Vitro Radiotracers Assays. Cell uptake of 8-[^3]H]PCV (0.2 μCi/ml) or 18 F-FHBG (5–10 μCi/ml) was performed after induction of hybrid proteins with doxycycline as described in figure legends (11). Data were expressed as moles nucleoside analogue/mg P/mnm, where mg P refers to mg cell protein and nm is concentration of nucleoside analogue in the extracellular space (14).

Western Blotting. Expression of Gal4-BD-p53, VP16-Tag, and VP16-CP hybrid proteins in cells and tumors was detected by Western blotting with primary antibodies to Gal4-BD and VP16 (Clontech, Palo Alto, CA) as described (10, 15).

Fluorescence Microscopy. Cultured cells and tumor specimens were processed for fluorescence microscopy as described previously (11, 16).

Animal Studies. All of the animal procedures were approved by the Washington University Medical School Animal Studies Committee. Gal4-BD-p53/VP16-TAg subclone 453 or 478 cells (1.2–1.8 × 10⁶) or Gal4-BD-p53/VP16-CP cells (1 × 10⁶) were injected s.c. into each axilla of adult Ncr nu/nu mice (Taconic, Germantown, NY). After ~5 mm tumors developed, mice were treated with i.p. injections of doxycycline to induce hybrid proteins. Imaging was performed 48 h after administration of various doses of doxycycline or at various times after injection of 60 μg doxycycline/g body weight in PBS as detailed in figure legends. Control animals received PBS alone.

For microPET imaging (Concorde Microsystems, Knoxville, TN), mice were lightly anesthetized with isoflurane before tail vein injection of 18 F-FHBG (∼50–170 μCi) and allowed to recover. One h after injection of radiotracer, mice were again anesthetized with isoflurane, placed supine within the microPET scanner, and imaged (10–20 min acquisition time; 1 bed position; filtered-back projection reconstruction; isotropic image resolution 1.8 mm). MicroPET images were corrected for decay, but not attenuation, and ROI were analyzed as described previously (10). VP16-CP tumors could not be detected by imaging with 18 F-FHBG, so ROI counts were obtained from tissues at the expected position of this tumor in each animal. Data for accumulation of 18 F-FHBG on serial microPET images were expressed as SUVs, which represent the counts per gram of tissue divided by injected dose of radioactivity per gram of animal weight (17). In TK-transduced tumors, 18 F-FHBG shows prolonged retention over 2–5 h, thereby enabling facile SUV analysis that is not critically time-dependent (18).

For biodistribution studies, animals were anesthetized with metofane before tail vein injection of 18 F-FHBG (∼15 μCi). Tumor and selected organs were harvested from animals 1 h after injection of radiotracer and assayed for radioactivity (Cobra II; Beckman). Content of 18 F-FHBG was expressed as percentage of injected dose/g of tissue (19). Tumor content of 18 F-FHBG was normalized to accumulation of radiotracer in the heart, an organ that does not accumulate significant amounts of 18 F-FHBG (18).

Statistics. Data are presented as mean values ± SE. Pairs were compared by Student’s t test (20); values of P ≤ 0.05 were considered significant.

RESULTS

Imaging Two-Hybrid System. We developed recently a tetra-cyccline-inducible two-hybrid system that enables interactions between the p53 tumor suppressor protein and large TAg from SV40 virus to be detected by microPET and fluorescence imaging. Our initial studies showed that association of p53 and TAg can be imaged in living mice, using tumor xenografts of HeLa cells stably transfected with all of the components of the inducible two-hybrid system. However, we did not investigate whether imaging provides only a binary response to protein interactions or whether microPET imaging can differentiate among relative amounts of interacting proteins in living animals. If the two-hybrid system produces graded responses to protein interactions, then in vivo imaging potentially could be used to investigate a wide variety of biological hypotheses, such as quantifying effects of post-translational modifications that alter affinity between two proteins.

From our original HeLa p53/TAg transfectants, we isolated subclones of cells that express various levels of the mTK-EGFP reporter enzyme in response to treatment with doxycycline and expression of interacting proteins. We selected two different subclones, referred to as TAg 453 and TAg 478 cells, with intermediate and high expression of mTK-EGFP, respectively, in response to induction of hybrid p53 and TAg. As a negative control pair of hybrid proteins that do not interact, we used a cell line that expresses Gal4-BD-p53 and VP16-CP (CP cells). These cell lines were used to additionally characterize the imaging two-hybrid system in cultured cells and in living mice.

Response of the Imaging Two-Hybrid System to Increasing Amounts of Interacting Proteins in Cultured Cells. To regulate expression of hybrid-positive and -negative interacting proteins, we treated TAg 453, TAg 478, and CP cells for 48 h with increasing amounts of doxycycline. By Western blotting, treatment with 100 ng/ml doxycycline induced immunodetectable amounts of Gal4-BD-p53 in all of the cell lines (Fig. 1A). Levels of Gal4-BD-p53 increased in response to higher doses of doxycycline up to 1000 ng/ml, the highest concentration tested. Overall, amounts of immunodetectable Gal4-BD-p53 were comparable among all three of the cell lines, although levels in TAg 453 cells were relatively lower at 100 ng/ml and slightly higher at 1 μg/ml doxycycline. Dose-dependent increases in immunodetectable VP16 hybrid proteins also were observed in all of the cell lines. In TAg 453 and 478 cells, VP16-TAg was identified first at 10 ng/ml doxycycline, whereas expression of VP16-CP could be faintly detected after treatment with 3 ng/ml doxycycline. At higher doses of drug, expression of VP16-TAg was comparable in both cell lines, whereas relative amounts of VP16-CP were slightly lower in CP cells. Discordance between concentrations of doxycycline needed to induce immunodetectable amounts of Gal4-BD-p53 versus VP16 hybrid proteins may be because of degradation of p53 by the ubiquitin-proteasome system. HeLa cells, which are transformed by human papillomavirus, ubiquitinate p53 through the viral E6 and cellular E6-associated protein (E6AP), thereby opposing expression of hybrid p53 from the tetracycline-responsive promoter (21, 22).

To determine expression of the mTK-EGFP reporter gene after induction and interaction of hybrid proteins, we incubated parallel cultures of doxycycline-treated cells with [3 H]PCV, a nucleoside analogue that is phosphorylated by TK (10, 23, 24). Phosphorylated [3 H]PCV is trapped within cells, whereas unphosphorylated [3 H]PCV is not retained within cells after transfer to radiotracer-free medium (11). Because net cell content of [3 H]PCV is proportional to amounts of reporter enzyme, accumulation of [3 H]PCV can be used to quantify interactions of hybrid proteins in the imaging two-hybrid system. In response to increasing concentrations of doxycycline, both TAg 453 and 478 cells showed dose-dependent increases in content of [3 H]PCV (Fig. 1B). In TAg 453 cells, treatment with 10 ng/ml doxycycline induced significant activity of the reporter protein (P < 0.001), and maximal cell content of nucleoside analogue occurred at 300 ng/ml doxycycline. By comparison, accumulation of [3 H]PCV above background levels was detected at 3 ng/ml doxycycline in TAg 478 cells (P < 0.001). Peak levels of [3 H]PCV in TAg
478 cells were detected at 100 ng/ml doxycycline, a point at which cell content of nucleoside analogue was ~400-fold greater than CP cells. Cell content of [3H]PCV in TAg 478 cells was greater than TAg 453 cells at all of the concentrations of drug, and peak accumulation of [3H]PCV in TAg 478 cells was ~2-fold greater than in TAg 453 cells. Activity of the mTK-EGFP reporter decreased in TAg 478 cells incubated with concentrations of doxycycline >100 ng/ml. For both TAg cell lines, activity of the mTK-EGFP reporter could be detected at concentrations of doxycycline that did not produce immunodetectable amounts of protein by Western blotting, thereby showing the relatively greater sensitivity of the radiotracer assay for monitoring protein interactions with the imaging two-hybrid system. Accumulation of [3H]PCV in CP cells remained essentially at background levels independent of doxycycline treatment and induction of hybrid proteins.

To analyze the enhanced GFP (EGFP) component of the reporter protein, we used fluorescence microscopy to monitor interactions between hybrid p53 and TAg. In TAg 478 cells, faint expression of GFP above background autofluorescence could be detected in cells treated with 10 ng/ml doxycycline, which is slightly higher than the 3 ng/ml concentration of drug needed to detect protein interactions with [3H]PCV (Fig. 1C). Dose-dependent increases in GFP intensity were observed qualitatively by fluorescence microscopy, and expression of GFP at a given dose of doxycycline appeared relatively uniform in all of the cells in the population. Unlike the radiotracer assay with [3H]PCV, qualitative expression of GFP in TAg 478 cells did not decrease in cells treated with 1000 ng/ml doxycycline. Potentially, this difference could be because of qualitative evaluation of GFP expression in a limited number of cells compared with quantitative measurement of radiotracer accumulation in an entire population of TAg 478 cells. TAg 453 cells also showed dose-dependent increases in reporter gene expression with GFP first detected at 100 ng/ml doxycycline (data not shown). Only background autofluorescence was evident from CP cells under any treatment condition, consistent with data for accumulation of [3H]PCV (data not shown). Overall, these data show that expression of the mTK-EGFP reporter gene generally corresponds to relative changes in amounts of positive interacting hybrid proteins induced by doxycycline.

**Time-dependent Regulation of the Imaging Two-Hybrid System.** Having demonstrated that the imaging two-hybrid system shows graded responses to various levels of interacting proteins, we then determined the kinetics for expression of the mTK-EGFP reporter gene after induction of hybrid interacting proteins p53 and TAg. As shown in Fig. 1A, hybrid proteins were undetectable in the absence of doxycycline. After incubation with 1 μg/ml doxycycline, Gal4-BD-p53 and VP16-hybrid proteins in all of the cells lines were detected.
readily after 4 h of incubation, the earliest time point investigated (Fig. 2A). Amounts of Gal4-BD-p53 increased slightly over time, reaching peak levels after 48 h of incubation with doxycycline. By comparison, time-dependent increases in protein expression were more apparent for VP16-hybrid proteins, and levels of these proteins were greatest after 24 h of induction with doxycycline. Relative amounts of hybrid p53 and TAg were greater in TAg 478 cells than the TAg 453 subclone.

We quantified expression of the mTK component of the reporter gene by measuring cell content of [3H]PCV in parallel cultures of doxycycline-treated cells. In TAg 453 cells, incubation with doxycycline for 24 h significantly increased accumulation of PCV above values in these cells in the absence of drug, whereas cell content of nucleoside analogue in TAg 478 cells was significantly greater after only 8 h (P < 0.01; Fig. 2B). Both positive interacting cell lines showed time-dependent increases in reporter function through 48 h, at which time accumulation of [3H]PCV in TAg 478 and 453 cells was ~370- and 110-fold greater than CP cells, respectively (P < 0.001). At all of the time points, cell content of [3H]PCV was greater in TAg 478 than in TAg 453 cells. Again, accumulation of [3H]PCV in CP cells was not significantly greater than background without or with doxycycline treatment.

Fluorescence microscopy of TAg 478 cells showed detectable expression of GFP from the mTK-EGFP reporter after 8 h of doxycycline treatment, which corresponds to the earliest time point detected with [3H]PCV (Fig. 2C). At the 8-, 12-, and 24-h time points, expression of GFP was heterogeneous among individual cells, as evidenced by foci of cells with relatively greater fluorescence. Qualitatively, time-dependent increases in GFP were detected through 72 h of incubation with doxycycline, and expression of fluorescent reporter protein also became more uniform in the population of treated cells. Kinetics of GFP expression in TAg 453 cells also paralleled data for...
accumulation of [\(^{3}H\)]PCV, whereas CP cells had no detectable fluorescence from GFP (data not shown). Collectively, these data demonstrated time-dependent expression of the mTK-EGFP reporter after induction of hybrid proteins with doxycycline, and confirmed that reporter activity in cultured cells differed among TAg 453, TAg 478, and CP lines. Therefore, these cells provided a model system to determine whether in vivo imaging could detect and quantify graded responses to various amounts of positive and negative interacting proteins.

**In Vivo Response of the Two-Hybrid System to Increasing Amounts of Hybrid Proteins.** To enable imaging of protein interactions in vivo, we formed tumor xenografts of TAg 453, TAg 478, and CP cells in nu/nu mice. When palpable tumors (<0.5 cm) developed, we treated mice with 6, 30, or 60 \(\mu\)g doxycycline i.p. for 48 h to induce hybrid positive and negative interacting hybrid proteins (\(n = 1\) for each dose of drug). Expression of the mTK-EGFP reporter gene in living mice in response to protein-protein interactions was detected by tail vein injection of \(\sim 50\) \(\mu\)Ci of \(^{18}\)F-FHBG (13), a nucleoside analogue that is phosphorylated by mTK and retained within cells (10). We performed whole-body microPET imaging of mice 1 h after injection of \(^{18}\)F-FHBG, corresponding to the in vivo kinetics for monitoring TK expression with a structurally related nucleoside analogue (25). Transaxial reconstructions of microPET data showed accumulation of \(^{18}\)F-FHBG in both TAg tumors (Fig. 3A), whereas content of radiotracer in CP tumors was not detectable above background accumulation in adjacent tissue (data not shown). Images also showed a variable amount of \(^{18}\)F in spine, which reflected defluorination of \(^{18}\)F-FHBG (18). \(^{18}\)F accumulates in bone and has been used for PET studies of bone metabolism and metastases (26, 27). After defining ROI around each TAg tumor, accumulation of \(^{18}\)F-FHBG was determined from SUVs (17). Previous research has shown that tissue content of radiotracer correlates highly with SUV values derived from PET images (28). CP tumors could not be detected with \(^{18}\)F-FHBG. Therefore, we localized CP tumors based on anatomical landmarks on each mouse, and quantified SUV from a ROI defined in muscle and tissue at the appropriate site. Muscle and skin have been shown previously to accumulate very low amounts of \(^{18}\)F-FHBG that essentially are at background levels of radioactivity (29). SUV values show that accumulation of \(^{18}\)F-FHBG in TAg 453 and TAg 478 tumors increased in response to increasing concentrations of doxycycline, whereas the low content of radiotracer in muscle was unaffected by the inducing drug (Fig. 3B). For a given dose of doxycycline, SUV for the TAg 478 tumor was slightly greater than the TAg 453 tumor. At the 60 \(\mu\)g/g dose of doxycycline, SUV values for TAg 453 and TAg 478 tumors were \(~10-\) and \(~13\)-fold greater than background, respectively.

To confirm data from microPET imaging, we also performed biodistribution studies on nu/nu mice bearing xenografts of the three tumor cell lines. Studies were performed as described for microPET imaging, except that each animal was injected with \(~15\) \(\mu\)Ci \(^{18}\)F-FHBG (\(n = 3\) mice per concentration of doxycycline). One h after injection of radiolabeled nucleoside analogue, mice were sacrificed, and tumor and tissue content of radioactivity were assayed directly by gamma counting. Data for tumor accumulation of \(^{18}\)F-FHBG were expressed as percentage of injected dose per g of tissue and normalized to content of radiotracer in the heart, an organ that does not phosphorylate and accumulate FHBG (18). Similar to SUV data from microPET images, the biodistribution study showed that content of radiotracer in TAg tumors increased with increasing amounts of doxycycline, whereas amounts of retained \(^{18}\)F-FHBG in CP tumors were unaffected by inducing hybrid proteins (Fig. 3C). In mice treated with 60 \(\mu\)g/g doxycycline, content of radiotracer in TAg 453 and TAg 478 tumors was \(~4-\) and 7-fold greater than CP tumors, respectively (\(P < 0.005\)). These data demonstrate that the imaging two-hybrid system produces a graded, rather than binary, response to differing relative amounts of interacting proteins in vivo.

We used fluorescence microscopy of excised tumor specimens to qualitatively assess expression of GFP from the reporter gene in response to interacting hybrid proteins in vivo. For all of the tumors, very low levels of fluorescence were detected in the absence of doxycycline (Fig. 3D). Relative amounts of GFP in TAg 478 tumors increased in response to induction of hybrid proteins with increasing concentrations of doxycycline administered to mice. In vivo expression of GFP was heterogeneous in TAg 478 tumors, suggesting that one or more transgenes for the two-hybrid system was stochastically silenced in some tumor cells (30). Alternatively, individual cells within a tumor may be exposed to differing amounts of doxycycline. Regardless of the etiology, heterogeneous activation of the reporter contributes in part to loss of signal from positive interacting proteins when the two-hybrid system is translated from cell culture to living mice. TAg 453 tumors showed a similar pattern of GFP expression, although fluorescence intensity was qualitatively lower than TAg 478 tumors (data not shown). As expected, doxycycline induction of negative interacting hybrid p53 and CP did not produce GFP in CP tumors (data not shown).

To verify that treatment with doxycycline induced hybrid proteins in vivo and to assess relative differences in amounts of protein in each tumor, we excised tumor specimens and used Western blotting to detect hybrid proteins in tumor lysates. In response to increasing concentrations of doxycycline, greater expression of Gal4-VP16 was present in each tumor (Fig. 3E). Somewhat lower amounts of hybrid p53 were detected in TAg 453 tumors. VP16 hybrid proteins also showed a small increase in levels of expression corresponding to greater doses of administered doxycycline. Again, amounts of hybrid VP16-TAg were slightly lower in TAg 453 tumors relative to TAg 478. The Western blots also confirm expression of hybrid proteins in CP tumors, demonstrating that low reporter activity is attributable to absence of protein interaction rather than lack of expression of hybrid proteins. Overall, these data show that microPET imaging can detect and quantify relative differences in amounts of interacting hybrid proteins in living mice.

**In Vivo Response of the Two-Hybrid System to Increasing Time of Expression and Interaction of Hybrid Proteins.** In cultured cells, expression of the mTK-EGFP reporter gene increased in a time-dependent fashion in response to induction of hybrid interacting proteins with doxycycline. To determine whether these differences in reporter activity could be monitored in vivo, we established xenografts of TAg 453, TAg 478, and CP cells in nu/nu mice, and treated animals with i.p. injections of 60 \(\mu\)g/g doxycycline for 13, 29, or 53 h before microPET imaging (\(n = 1\) animal for each time point). Coronal reconstructions of microPET scans showed accumulation of \(^{18}\)F-FHBG in TAg 453 and TAg 478 tumors, but no radioactivity above background levels could be detected in the expected sites of CP tumors (Fig. 4A). Large amounts of \(^{18}\)F-FHBG were observed in gallbladder and intestine, reflecting clearance of radiotracer through the hepatobiliary system (29). SUV values demonstrated time-dependent enhancement of \(^{18}\)F-FHBG in each TAg tumor, showing that induction of interacting pairs of hybrid proteins for increasing periods of time produced greater expression of the mTK-EGFP reporter gene (Fig. 4B). For TAg 478 tumors, only a small increment in SUV was measured between the 12- and 24-h time points. The TAg 478 tumor grew poorly and was very small in the mouse imaged after 24 h of doxycycline, which may account for the unexpectedly low content of radiotracer in this tumor. At all of the other time points, SUVs for accumulation of \(^{18}\)F-FHBG were higher in TAg 478 than TAg 453 tumors, showing that in vivo imaging could detect and
quantify relative differences in reporter gene expression comparable to that produced in cultured cells. As observed previously, background accumulation of nucleoside analogue in the CP tumor, as defined by tissue at the anatomical site of this tumor, was not affected by doxycycline.

We also performed biodistribution studies in mice bearing xenografts of the various tumor cells after treatment with doxycycline for various periods of time as described for microPET imaging \((n = 2–4\) mice/treatment time). In the absence of doxycycline, accumulation of \(^{18}\text{F}\)-FHBG was low and did not differ among tumor types.

Fig. 3. Doxycycline dose-dependent regulation of protein interactions in vivo. A, microPET imaging. Mice bearing tumor xenografts of TAg 453, TAg 478, and CP cells were treated with i.p. injection of various concentrations of doxycycline administered every 8 h over 48 h for a total of seven doses. MicroPET imaging was performed 1 h after tail vein injection of \(-50\ \mu\text{Ci} of \(^{18}\text{F}\)-FHBG. Transaxial images through TAg 453 (white arrow) and TAg 478 tumors (yellow arrow) are shown. A variable amount of activity was observed in vertebral bodies (+) depending on levels of defluorination of nucleoside analog occurring within a given mouse (18). B, SUV. ROIs were determined from microPET images and used to calculate SUV for each tumor \((n = 1\) for each concentration). SUVs in muscle within the expected site of CP tumors were used to determine background accumulation of radioactivity, because CP tumors could not be visualized with \(^{18}\text{F}\)-FHBG. C, biodistribution studies. Mice were treated with doxycycline \((n = 3–4\) for each concentration) to induce hybrid proteins as described above and then injected with \(-15\ \mu\text{Ci}\) of \(^{18}\text{F}\)-FHBG. Tumors and organ samples were removed 1 h after administration of radiotracer, and content of \(^{18}\text{F}\) was determined by gamma counting. Data are expressed as tumor:heart ratios of percentage of injected radioactivity per g of tissue. D, ex vivo fluorescence microscopy of tumor specimens. Expression of GFP from the mTK-EGFP reporter was analyzed in cryosections of excised tumors. Representative images \((\times 400\) magnification) are shown for TAg 478 tumors taken from mice treated with various concentrations of doxycycline. E, expression of hybrid proteins. Excised tumors were lysed, and expression of hybrid proteins in 150 \(\mu\text{g}\) of total lysate was detected with antibodies to Gal4-BD (left panel) and VP16 (right panel).
After inducing hybrid p53 and TAg with doxycycline, both TAg 453 and TAg 478 tumors showed time-dependent increases in content of radiotracer. After 48 h of doxycycline treatment, accumulation of $^{18}$F-FHBG was ~5- and 10-fold higher in TAg 453 and TAg 478 tumors, respectively, compared with CP ($P < 0.01$). In mice treated with doxycycline, content of radiotracer in TAg 478 cells was significantly greater than TAg 453 cells at all of the time points ($P < 0.01$).

To confirm that the GFP component of the reporter protein also was induced in vivo, we analyzed sections of excised tumors by fluorescence microscopy. In TAg cells, interactions between hybrid p53 and TAg produced time-dependent enhancement of GFP (Fig. 4D; data not shown). Again, heterogeneous expression of GFP was observed in TAg tumors after treatment of mice with doxycycline, whereas no fluorescence above background levels was detected without doxycycline. CP tumors did not express GFP under any conditions. Western blotting of excised tumor specimens showed that expression of hybrid proteins, especially VP16 hybrids, increased in mice treated for longer periods of time with doxycycline (Fig. 4E). Overall, these data confirmed that inducing hybrid interacting proteins produced graded increases in the mTK-EGFP reporter that could be measured in living mice with microPET imaging.

**DISCUSSION**

A key step in determining biological functions of any protein is determining its interactions with other proteins in signal transduction and biochemical pathways. Binding and dissociation of proteins frequently are dynamic processes, and, thus, protein-protein interactions are modified by physiological and pathophysiologic conditions that exist in intact organisms. Development and application of noninvasive imaging techniques for monitoring protein-protein interactions would enable real-time investigations of mechanisms that regulate formation of protein complexes in vivo. In our current study, we have additionally characterized a two-hybrid system for microPET and fluorescence imaging of protein-protein interactions in living mice.

An important conclusion from the current data are that the imaging two-hybrid system generally responded in a proportional fashion to increasing amounts of hybrid proteins in cultured cells and in living mice. Using a tetracycline-inducible system, we regulated expression of hybrid p53 and TAg in stably transfected cells and tumor xenografts. Amounts of hybrid proteins increased in response to increasing doses of doxycycline, and greater levels of interacting hybrid proteins typically enhanced expression of the mTK-EGFP reporter. Doxycycline-dependent enhancement of reporter activity was detected both in cell culture and living mice, as monitored by the TK and fluorescence imaging systems.

**Fig. 4.** Time-dependent induction of protein interactions in living mice. A, microPET imaging. Mice bearing tumor xenografts of TAg 453, TAg 478, and CP cells were treated with i.p. injections of 60 µg/g doxycycline for 13, 29, and 53 h before microPET imaging ($n = 1$ mouse for each time point). Doses ($n = 2, 4, and 7$ total doses of doxycycline for mice treated for 13, 29, and 53 h, respectively) were administered every 8 h. Coronal images show accumulation of $^{18}$F-FHBG in TAg 453 (white arrow) and TAg 478 (yellow arrow) tumors. * marks anatomical location of CP tumor. Normal excretion of $^{18}$F-FHBG into gall bladder and intestine is seen in the lower part of each image. B, SUVs. SUV from microPET images are shown for each TAg tumor ($n = 1$ for each time point). SUV from muscle was used, because CP tumors could not be visualized on microPET images. C, biodistribution studies. Mice ($n = 1, 3, 2, and 4$ mice for 0, 13, 29, and 53 h of doxycycline treatment, respectively) were treated with doxycycline as described in A to induce hybrid proteins, and content of $^{18}$F-FHBG in excised tumors and normal tissues was quantified as described in Fig. 3. D, ex vivo fluorescence microscopy of tumor specimens. Expression of GFP from the mTK-EGFP reporter was analyzed in cryosections of excised tumors. Representative images ($\times 400$ magnification) are shown for TAg 478 tumors taken from mice treated with doxycycline for the indicated periods of time. E, expression of hybrid proteins. Western blotting was used to detect hybrid proteins in CP, TAg 453, and TAg 478 tumors in mice treated with doxycycline for the indicated times, using antibodies to Gal4-BD (left panel) and VP16 (right panel).
GFP components of the fusion reporter gene. Activity of the reporter gene remained at background levels in the absence of doxycycline or in cells that expressed noninteracting p53 and CP hybrid proteins, demonstrating that expression of the reporter gene required interacting proteins. Overall, the data indicated that the imaging two-hybrid system produced a graded, rather than binary, response to various amounts of interacting proteins in cultured cells and in vivo.

Because two-hybrid assays rely on transcription of a reporter gene, a lag exists between binding of p53 to TAg and subsequent expression of the reporter. In cultured cells, function of mTK-EGFP could be detected 8 h after inducing interacting proteins with doxycycline. Expression of the reporter increased in a time-dependent fashion through 48 h of incubation with doxycycline, indicating that continued binding of p53 to TAg produced increasingly greater amounts of mTK-EGFP. By microPET imaging, similar time-dependent increases in reporter activity were seen in response to expression of hybrid p53 and TAg. Protein-protein interactions were detected after treatment of animals with doxycycline for 13 h, which was the earliest time point examined by imaging. Thus, kinetics of the imaging two-hybrid system potentially limit the types of biological hypotheses that can be investigated in vivo. Transient binding and dissociation of proteins may not produce sufficient differences in levels of reporter to be detected by noninvasive imaging. Therefore, two-hybrid imaging may be most relevant for studies in which changes in steady-state protein interactions are biologically relevant.

In general, increased expression of positive interacting hybrid proteins produced greater activity of the reporter protein in both cultured cells and living mice. However, there was not always a direct linear relationship between these parameters. Cell culture assays with TAg 478 cells showed that accumulation of $[^3H]PCV$ decreased at higher concentrations of doxycycline. By comparison, fluorescence from GFP continued to increase at higher concentrations of doxycycline, suggesting that amounts of the fusion reporter protein did not diminish. Potentially, cellular accumulation of $[^3H]PCV$ was limited by transport of nucleoside analogue into cells and/or intracellular delivery of substrate to the reporter protein. Most mammalian cells express a variety of different isosferms of nucleoside transporters at the cell membrane, many of which are regulated differentially (31). In response to transient induction of protein-protein interactions and expression of the reporter gene, capacity of nucleoside transporters might be insufficient to provide saturating amounts of nucleoside analogue for phosphorylation by mTK-EGFP. Additional research is needed to determine effects of transport and phosphorylation of nucleoside analogues on overall accumulation of radiolabeled substrate in cells that express TK reporters.

The imaging two-hybrid system showed graded responses to interacting proteins both in cultured cells and in living mice, although the in vitro and in vivo data cannot be compared directly, because we did not determine the concentration of doxycycline within each tumor after systemic delivery of drug. Furthermore, as demonstrated by the time-dependent effects of doxycycline in cell culture and mice, small increases in amounts of interacting proteins produced relatively large increases in expression and function of the mTK-EGFP reporter. In particular, time-dependent changes in levels of Gal4 BD-p53 were modest. Differences among expression of hybrid proteins and activity of mTK-EGFP likely reflect the overall complexity of the imaging two-hybrid system, in which detection of protein-protein interactions is dependent on a variety of interdependent factors: induction, stability, and association of hybrid proteins; expression of reporter gene; and pharmacokinetics and pharmacodynamics of the radiolabeled nucleoside and reporter protein in vivo. Nevertheless, the data show that the imaging two-hybrid system is not limited to binary responses to interacting proteins despite the overall complexity of the method for interrogating protein-protein interactions in the context of a living animal.

Although we have used the strong, constitutive interaction between p53 and TAg to develop and characterize the imaging two-hybrid system, we envision that noninvasive imaging could be used for in vivo studies of relative differences in binding affinity among proteins or to investigate proteins with weaker association. Previous research in cultured cells has demonstrated that two-hybrid assays can distinguish relative differences in binding affinity among interacting proteins based on differences in reporter activity (32). Accumulation of radiolabeled nucleoside analogue correlates highly with amounts of TK enzyme both in cell culture and living animals (25), and our current data show that microPET imaging of protein-protein interactions can distinguish differences in reporter activity in living animals. Therefore, we anticipate that two-hybrid imaging could be used to quantify relative differences in binding affinities or amounts of interacting proteins. As an example, phosphorylation and dephosphorylation of proteins regulate reversible interactions between proteins that control cell-cycle transitions. Additional studies are needed to determine the limits of two-hybrid imaging for quantifying interactions between proteins with weak interactions and to enhance sensitivity of the system for extending the range of interacting proteins that can be analyzed in living animals.

In summary, our data demonstrate that microPET and fluorescence imaging of protein-protein interactions respond in proportional fashion to increasing amounts of interacting proteins in vivo. Noninvasive imaging will allow protein complexes to be studied in the context of the regulatory pathways that modulate protein interactions in living animals. By enabling serial studies of the same animal over time, noninvasive imaging can overcome animal-to-animal variations in experimental data, potentially revealing small differences in steady-state protein interactions. In addition, the two-hybrid imaging system will facilitate studies of pharmacokinetics, pharmacodynamics, and overall efficacy of drugs targeted to specific protein-protein interactions.

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