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A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins

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ABSTRACT

A number of proteins have been rendered functionally oestrogen-dependent by fusion with the hormone-binding domain of the oestrogen receptor. There are, however, several significant disadvantages with such fusion proteins. First, their use in cells *in vitro* requires phenol red-free medium and laborious stripping of steroid hormones from serum in order to avoid constitutive activation. Secondly, control of oestrogen receptor fusion proteins *in vivo* is precluded by high endogenous levels of circulating oestrogens. Thirdly, the hormone-binding domain of the oestrogen receptor functions as a hormone-dependent transcriptional activation domain making interpretation of fusions with transcription factors problematical. In order to overcome these drawbacks we have used a transcriptionally inactive mutant of the murine oestrogen receptor which is unable to bind oestrogen yet retains normal affinity for the synthetic ligand, 4-hydroxytamoxifen. When the hormone-binding domain of this mutant oestrogen receptor is fused to the C-terminus of the c-Myc protein, Myc-induced proliferation and apoptosis in fibroblasts becomes dependent on 4-hydroxytamoxifen, but remains refractory to 17β-oestradiol.

INTRODUCTION

Several intracellular proteins have been rendered functionally hormone-dependent by fusing them with the hormone binding domain (HBD) of steroid receptors [reviewed in (1,2)]. This strategy has been used successfully to generate conditional forms of several viral and cellular transcription factors, including adenovirus E1A (3,4), HIV Rev (5), c-Myc (6), v-Myb (7), various members of the Fos family (8,9), MyoD (10), p53 (11), C/EBP (12), v-Rel (13), GATA-1, 2 and 3 (14) and Gal4-VP16 (15,16) as well as protein kinases including c-Abl (17) and Raf1 (18). Thus, the strategy of HBD fusion appears generally applicable to different functional types of protein.

HBD-fusion proteins are probably inactive in the absence of ligand because they are complexed with a variety of intracellular polypeptides, of which Hsp90 is the prototype [reviewed in (19,20)]. Ligand binding releases the receptors from the inhibitory complexes. Five vertebrate steroid receptors, the glucocorticoid, mineralocorticoid, androgen, progesterone and oestrogen receptors, are known to be associated with, and inactive in, such complexes. Of these five receptors, the hormone binding domain of the human oestrogen receptor (ER) has been most widely used as a heterologous regulatory domain: the ligand, 17β-oestradiol (E2), is readily available, relatively cheap and many cell types lack endogenous oestrogen receptor. However, the HBD of ER has significant practical drawbacks as a switch. First, it possesses an inherent ligand-dependent transactivation activity, referred to as TAF-2 or AF-2 (21–23) which may, therefore, contribute to the total transcriptional activity of the fusion protein. As a consequence interpretation of results, particularly where the heterologous partner has weak transcriptional activity, is not always straightforward (8). Furthermore, using the HBD of ER to regulate the activity of transcriptional repressors is unlikely to be feasible. Secondly, most *in vitro* experimental systems use media containing phenol red, a weak agonist of ER (24), and serum, which usually contains oestrogens. These problems are partly mitigated by the use of the low affinity V400 mutant hormone binding domain of ER (25,26). However, use of this mutant still requires use of culture media free of phenol red and the removal of oestrogens from serum by the laborious process of stripping with charcoal–dextran which, in any event, can still induce low level activation of the wild type oestrogen receptor HBD (27,28) and raises the danger of batch variation and depletion of additional serum components other than steroid hormones. Finally, the high levels of oestrogens present in plasma preclude the use of ER-based switches in *in vivo* transgenic systems.

In order to overcome these drawbacks, we have made use of the mutant murine oestrogen receptor, G525R (27). This mutant no longer binds E2, possesses no TAF-2 transactivation activity, yet

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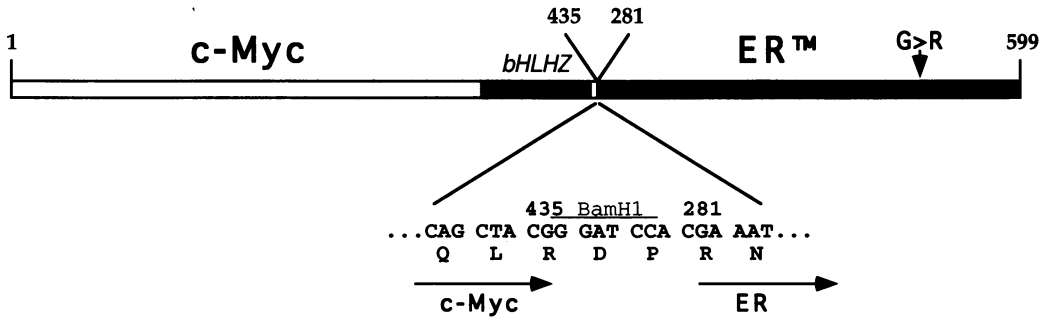

Figure 1. Construction of c-MycER™ chimaeric proteins. The mutant hormone binding domain of the murine oestrogen receptor (amino acids 281–599 containing a single amino acid change from glycine to arginine at position 525) was fused at the C-terminus of the human c-Myc protein (amino acids 1–435) to form c-MycER™. This fusion replaces the C-terminal four amino acids of c-Myc immediately adjacent to the basic-helix–loop–helix-leucine zipper (bHLHZ) with two amino acids (DP) generated by the insertion of a *Bam*HI site to facilitate fusion with ER™ (see Materials and Methods). Fusion of the ER™ fragment to the inactive c-Myc deletion mutant D106-143 generated D106-143c-MycER™.

remains responsive to activation by the synthetic steroid 4-hydroxytamoxifen (OHT) (27). To test the efficacy of the G525R mutant switch, we have fused an N-terminally truncated form of the G525R mutant oestrogen receptor (ER™) to the c-Myc protein. Myc proteins are involved in cell transformation, proliferation and apoptosis [reviewed in (29,30)]. In rodent fibroblasts that express a chimaeric protein comprising human c-Myc fused to the hormone binding domain (HBD) of the human oestrogen receptor (ER), these activities of Myc become strictly dependent upon the presence of exogenous 17β-oestradiol (E2) (6,31,32) . Here we show that the c-MycER™ fusion protein exhibits c-Myc activities which are tightly dependent upon OHT yet totally unresponsive to oestrogen.

MATERIALS AND METHODS

Construction of c-MycER™

DNA encoding amino acids 281–599 of the murine oestrogen receptor that includes a point mutation at amino acid 525, resulting in replacement of the Gly residue with Arg (MOR G525R), has been described (27). In order to facilitate fusion of this sequence to c-*myc* a *Bam*HI site was created at the 5′ end of MOR G525R. The polymerase chain reaction was used to amplify DNA encoding amino acids 281–339 of MOR G525R using the following primers: 5′-GTCGTCGACGGATCCAC-GAAATGAAATGGGTGCTTCAGG-3′ and 5′-TCTTCTAGA-AGGATCATATTCAGAATAG-3′. The *Bam*HI and the *Xba*I (equivalent to amino acid 339) sites are underlined, respectively. The amplified DNA was cloned into pBluescript KS+ (Stratagene), verified by sequencing and ligated to DNA encoding amino acids 340–599 of MOR G525R to form pBS+ER™ (oestrogen receptor$^{\text{Tamoxifen Mutant}}$). DNA encoding amino acids 1–435 of human c-*myc* was excised from pMV7MycER (6) and fused at the 5′ end of the ER™ sequence as shown in Figure 1. This fusion creates an in-frame *Bam*HI site at the point of fusion which introduces two extra amino acids (Asp–Pro) which are not present in either c-Myc or ER™ at this position. *D106-143c-MycER*™ was created in a similar way by fusing *D106-143c-myc* from pMV7D106-143MycER (6) to ER™. Subsequently an *Eco*RI fragment containing the entire c-*myc*-ER™ or *D106-143c-mycER*™ was cloned in the correct orientation into the *Eco*RI site of the retrovirus vector pBpuro (33).

Cell lines and antibodies

The retroviral vectors pBpuro, pBpuro c-*mycER*™ or pBpuro *D106-143c-myc-ER*™ were transiently transfected into the ecotropic retroviral packaging cell line GP+E. Recombinant retrovirus was harvested after 48 h and used to infect Rat-1 and Swiss 3T3 rodent fibroblasts in the presence of 8 μg/ml polybrene. Puromycin resistant clones (12 of each) were selected in the presence of 5 μg/ml puromycin and the cells maintained in DMEM (or phenol red-free DMEM) supplemented with 10% foetal bovine serum and 5 μg/ml puromycin. Cell lysates of representative clones were fractionated on 10% polyacrylamide–SDS gels and transferred to Immobilon-P (Millipore). Expression of c-MycER™ and D106-143c-MycER™ was determined by immunoprobing with the human c-Myc-specific monoclonal antibody, Myc1-9E10 (34). Bound antibody was detected with horseradish peroxidase-linked anti-mouse Ig (Amersham) and ECL (Amersham).

Determination of Myc-induced cell proliferation

Cells were rendered quiescent by growth to confluence and serum-deprivation for a further 2 days (Rat-1) or by culture of sub-confluent population in serum-free medium for 2 days (Swiss 3T3). 17β-oestradiol, 4-hydroxytamoxifen or serum was then added in the presence of 10 μM BrdU. Incorporation of BrdU was assessed by flow cytometry, as described previously (31), or by immunocytochemical analysis using anti-BrdU antibody (Sera-lab). Immunocytochemical analyses were conducted on two independent fields each containing 100 cells.

Determination of Myc-induced apoptosis

The rate of apoptosis following addition of 17β-oestradiol or 4-hydroxytamoxifen was determined by time-lapse videomicroscopy as previously described (31,35,36). The results are presented as cumulative cell deaths versus time.

RESULTS

Expression of the c-Myc MER G525R fusion protein

The mutant HBD of the murine ER (MOR G525R) has a 1000-fold lower affinity for oestrogen than the wild type HBD, whereas its affinity for 4-hydroxytamoxifen is unchanged (27).

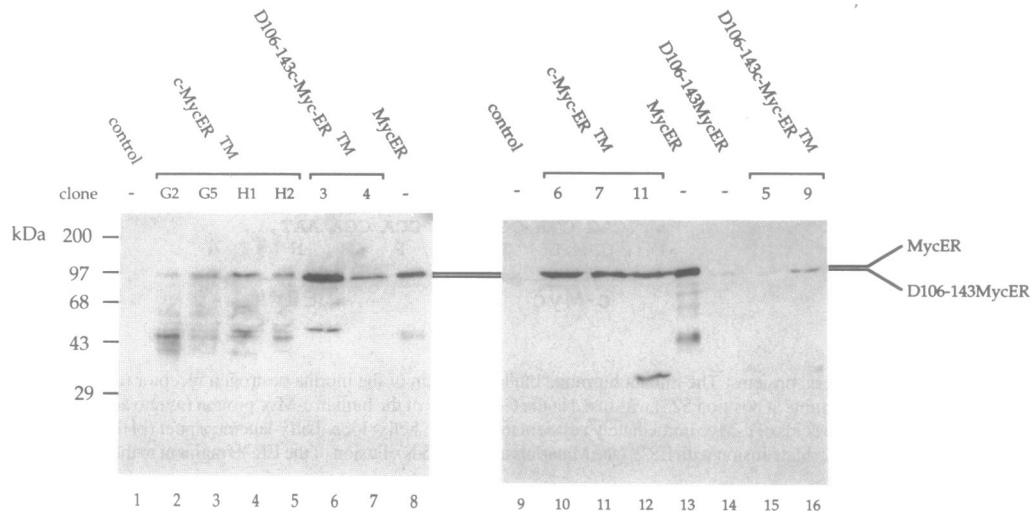

Figure 2. Expression of c-MycER™ and D106-143c-MycER™ in rodent fibroblasts. Protein lysates representing an equivalent number of cells (10^6) from individual puromycin-resistant clones of Rat-1/pBpuro (control, lane 1), Rat-1/c-MycER™ (lanes 2–5), Rat-1/D106-143c-MycER™ (lanes 6 and 7), Swiss 3T3/pBpuro (control, lane 9), Swiss 3T3/c-MycER™ (lanes 10–12), Swiss 3T3/D106-143c-MycER™ (lanes 15 and 16) or from established Rat-1/MycER (6) (lanes 8 and 13) or Rat-1/D106-143MycER (6) (lane 14) were fractionated on a 10% SDS–polyacrylamide gel and transferred to Immobilon-P membranes. The membranes were probed with Myc1-9E10 antibody, followed by sheep anti-mouse-HRP conjugate and ECL peroxidase substrate (Amersham). The positions of size markers in kDa are shown.

To test the biological responsiveness of MOR G525R, part of the domain was fused to either wild-type c-Myc (c-MycER™, Fig. 1) or the non-functional c-Myc deletion mutant D106-143 (D106-143c-MycER™). The chimaeric proteins were then expressed in the rodent cell lines Rat-1 and Swiss 3T3 by retroviral infection followed by puromycin selection. Twelve independent clones of each infected cell line were expanded and expression of c-MycER™ and D106-143c-MycER™ proteins determined by Western blotting. Figure 2 shows several representative clones of Rat-1 and Swiss 3T3 cells that constitutively express either c-MycER™ or D106-143c-MycER™. Levels of expression of the c-MycER™ chimaeras were similar in each clone and comparable to previously observed levels of expression in Rat-1 cells of c-Myc fused to the V400 mutant of human ER (31) (Rat-1/MycER; Fig. 2 and data not shown). Thus, both c-MycER™ and D106-143c-MycER™ proteins can be expressed in Rat-1 and Swiss 3T3 fibroblasts.

Tamoxifen-dependent MycER™-induced cell proliferation

Activation of MycER by 17β-oestradiol is sufficient to drive quiescent serum-deprived fibroblasts into the cell cycle (6,31). To determine the response of c-MycER™ to 17β-oestradiol (E2) and 4-hydroxytamoxifen (OHT), Rat-1/c-MycER™ and Swiss 3T3/MycER™ cells were rendered quiescent by culture in serum-free medium for 2 days and then treated with either 100 nM 17β-oestradiol or 100 nM 4-hydroxytamoxifen in the presence of 10 μM BrdU. After 18 h, cells were harvested, stained with propidium iodide and anti-BrdU antibody, and analysed by flow cytometry (Table 1). Although limited cell death was observed during the elapsed time of these experiments (see below), only data on cell proliferation is presented in Table 1. E2 failed to induce entry of quiescent Swiss 3T3/MycER™ or Rat-1/c-MycER™ cells into the cell cycle. Although in some experiments a slight stimulation of BrdU incorporation was observed in some clones of Rat-1/c-MycER™ and Swiss 3T3/MycER™ cells this was not a consistent finding and a similar stimulation was occasionally seen in control Rat-1/pBpuro and Swiss 3T3/pBpuro cells (Table 1). In contrast, addition of 100 nM OHT is mitogenic in both cell types, leading to substantial BrdU incorporation during S-phase. Time-lapse videomicroscopic observation of Swiss 3T3/c-MycER™ indicates that OHT is sufficient to keep these cells in cycle with an inter-mitotic time of ~19 h, similar to that of parental Swiss 3T3 cells growing exponentially in 10% foetal calf serum (data not shown). Induction of proliferation in c-MycER™ cells by OHT requires a functional Myc protein since cells expressing the deletion mutant D106-143c-MycER™ do not proliferate in the presence of OHT (Table 1). OHT does not exert any mitogenic effect on Rat-1 or Swiss 3T3 harbouring the empty control retrovirus pBpuro (Table 1). In addition, phenol red exhibited no mitogenic effect on quiescent Rat-1/c-MycER™ cells which had been previously cultured in phenol red-free DMEM (data not shown).

In order to determine the 'optimal' effective concentration of OHT for induction of mitogenesis by c-MycER™, quiescent Rat-1/c-MycER™ cells were treated with a range of OHT concentrations in the presence of BrdU and the proportion of cells entering S-phase (i.e. incorporating BrdU) at 18 h was determined in each case by flow cytometry. Maximal response was observed at a concentration of 100 nM OHT in the culture medium: higher concentrations led to no increase in the proportion of cells incorporating BrdU and concentrations <100 nM resulted in fewer cells entering S-phase and little, if any, mitogenic effect was seen <10 nM (data not shown). In analogous experiments, concentrations of E2 ≤1 μM failed to induce mitogenesis.

In summary, we conclude that fusion of c-Myc with the G525R mutant murine ER hormone-binding domain generates a conditional allele of c-Myc whose mitogenic effects are strictly dependent upon the presence of OHT yet refractory to E2.

Table 1. Induction of proliferation by c-MycER™ is dependent on 4-hydroxytamoxifen

Cell line	Percentage cells incorporating Brdu (18 h)			
	0% serum	100 nM E2	100 nM OHT	10% serum
Swiss 3T3/pBpuro control	1.74	8.78	0.72	95.44
Swiss 3T3/c-MycER™ (11)	10.0	15.2	57.5	63.7
Swiss 3T3/c-MycER™ (15)	ND	12.0	44.5	81
Swiss 3T3/D106-143c-MycER™ (12)	12.62	6.03	15.69	40.58
Rat-1/pBpuro control	7.08	11.87	6.79	64.29
Rat-1/c-MycER™ (G2)	5.98	8.21	29.73	ND
Rat-1/D106-143c-MycER™ (4)	13.37	12.12	13.81	53.51

Rat-1 or Swiss 3T3 fibroblasts expressing c-MycER™ or D106-143c-MycER™ were either grown to confluence and then cultured in serum-free medium for a further 2 days (Rat-1) or sub-confluent cultures transferred to serum-free medium for 2 days (Swiss 3T3). Parallel cultures of cells were then treated as indicated in the presence of 10 μM BrdU and the percentage of cells incorporating BrdU during the following 18 h determined by indirect immunofluorescence with an anti-BrdU antibody by flow cytometry (Rat-1) or by indirect peroxidase staining and counting stained cells in two randomly chosen fields of 100 cells (Swiss 3T3). The clone of cells used in these experiments is shown in parentheses.
ND = not done.

Tamoxifen-dependent Myc-induced apoptosis

Deregulated expression of Myc in the absence of survival cytokines (e.g. low serum) induces apoptosis in fibroblasts (31,36). Accordingly, functional activation of MycER with 17β-oestradiol rapidly induces apoptosis in serum-deprived fibroblasts (31,36). Induction of both proliferation and apoptosis appear to be mediated by the same sequence-specific DNA binding transcriptional activity of c-Myc (31,37). We tested the abilities of OHT and E2 to induce apoptosis in fibroblasts expressing c-MycER™. Quiescent, serum-deprived Rat-1/c-MycER™ cells rapidly undergo apoptosis in response to OHT, as demonstrated by time-lapse videomicroscopy (Fig. 3). The first cell deaths occur within 3 h of addition of OHT: the whole culture is typically dead within 60 h. The cell deaths we observe exhibit all the characteristics of apoptosis, namely nuclear condensation, membrane blebbing and fragmentation into apoptotic bodies (not shown). Degradation of cellular DNA into nucleosomal fragments, a frequent feature of apoptosis, was also evident (not shown). In contrast, fibroblasts expressing c-MycER™ exhibit very few cell deaths upon treatment with E2. This low level of apoptosis represents inherent low level cell death (i.e. not due to activation of c-MycER™) since control Rat-1 cells infected with the empty retrovirus also die at the same low rate in the presence or absence of E2 (Fig. 3), as do serum-deprived cells expressing the inactive mutant D106-143c-MycER™ in the presence or absence of OHT (data not shown). We also observe similar OHT-dependent apoptosis in Swiss 3T3/c-MycER™ (data not shown).

Thus, we conclude that expression of c-MycER™ induces apoptosis only in the presence of OHT and is unresponsive to E2.

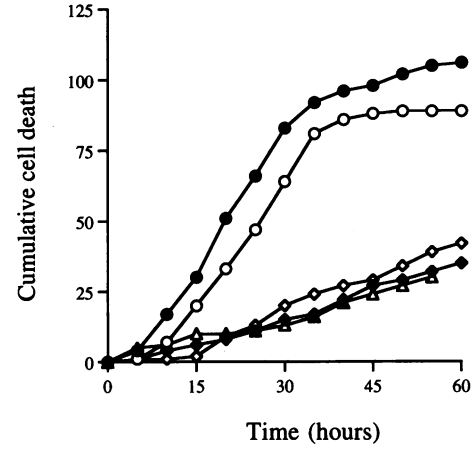

Figure 3. Induction of apoptosis by c-MycER™ is dependent on 4-hydroxytamoxifen. Quiescent Rat-1 c-MycER™ clones G2 (● and ◆) and G5 (○ and ◇), and Rat-1 cells infected with the empty pBpuro virus and treated with E2 (△) were supplemented with 100 nM 17β-oestradiol (◆ and ◇) or 100 nM 4-hydroxytamoxifen (● and ○). A field of ~100 cells was observed by time-lapse videomicroscopy and cumulative cell deaths plotted against time.

DISCUSSION

Fusion of many proteins with the hormone-binding domain of the oestrogen receptor has proved valuable in rendering their activity hormone-dependent. However, this strategy is compromised by a number of drawbacks. First, the presence of an inherent oestrogen-dependent transactivation activity in the HBD of ER confuses interpretations because any *trans*-activation that occurs could arise from this additional activity. Secondly, it is necessary to remove steroid hormones from foetal calf serum as well as phenol red from the medium used in the *in vitro* culture of cells. Thirdly, appreciable levels of oestrogens in plasma preclude the use of ER as a switch module to regulate transgenically targeted proteins.

To overcome these drawbacks we have used a previously described mutant of the murine oestrogen receptor HBD which, although unable to bind 17β-oestradiol, can still bind the synthetic analogue 4-hydroxytamoxifen (OHT). When fused to the c-Myc protein, the mutant ER™ HBD confers OHT dependence upon the resultant c-MycER™ fusion protein as induction of proliferation and apoptosis both absolutely require OHT. In contrast, 17β-oestradiol induces neither proliferation nor apopto-

sis. Moreover, both proliferation and apoptosis remain dependent on the integrity of the c-Myc protein since a deletion mutant which removes amino acids 106–143 of the Myc protein (D106-143c-MycER™) is unable to induce proliferation or apoptosis in response to OHT. Further studies indicate that co-transformation of rat embryo fibroblasts with c-*mycER*™ and an activated *ras* oncogene also requires OHT (J. Vlach and B. Amati, personal communication).

When compared with the oestrogen-responsive HBD of the human oestrogen receptor, which has been extensively used to regulate the activity of heterologous proteins to which it is fused, the mutant murine ER HBD (ER™) offers significant advantages. The ER™ possesses very low affinity for 17β-oestradiol, which has three important implications. First, OHT does not stimulate the TAF-2 *trans*-activation activity of ER™ (27), so avoiding problems of interpretation when studying transcription factor–HBD fusion proteins. Secondly, normal tissue culture media and untreated foetal calf serum may be used to culture cells harbouring ER™ fusion proteins without spuriously activating their function. Thirdly, chimaeric proteins carrying the ER™ should have great potential for use in *in vivo* experiments because they are refractory to the effects of endogenous oestrogens but responsive to pharmacologically attainable levels of 4-hydroxytamoxifen in plasma. Thus, a gene encoding, for example, a chimaeric c-MycER™ protein could be transgenically targeted to a particular tissue and the protein product functionally activated (and de-activated) by administration of Tamoxifen which is metabolised in the liver to 4-hydroxytamoxifen. By analogy, it might be possible to ablate the function of a particular protein by OHT-dependent activation of a dominant negative ER™ fusion expressed in transgenic animals. The effects and pharmacology of tamoxifen have been well characterised through its use in animal and human trials and although tamoxifen itself exerts a number of oestrogenic effects in certain normal tissues such as the endometrium and bone (38–41), these effects are unlikely to represent a problem for the majority of experiments. The ability to ectopically regulate the activity of the introduced chimaeric protein at any developmental stage offers a more refined approach than the standard methodologies of constitutively active generalised or tissue-specific expression of a transgene or complete gene ablation. Thus, the ER™ has great potential as a tool to investigate the biological function of a particular protein within the whole organism.

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