A chimeric Cre recombinase inducible by synthetic, but not by natural ligands of the glucocorticoid receptor

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ABSTRACT

We have developed a new ligand-dependent chimeric recombinase (Cre-GRdex) by fusing the site-specific Cre recombinase to the ligand binding domain (LBD) of a mutant human glucocorticoid receptor (GRdex). The synthetic glucocorticoid receptor (GR) ligands dexamethasone, triamcinolone acetonide and RU48486 efficiently induce recombinase activity in F9 murine embryonal carcinoma cells expressing constitutively Cre-GRdex. In contrast, no recombinase activity was detected in the absence of ligand or in the presence of the natural GR ligands cortisol, corticosterone or aldosterone. Moreover, physiological concentrations of these natural GR ligands do not affect Cre-GRdex recombinase activity induced by dexamethasone. Thus, as previously shown using Cre-oestrogen receptor (ER) fusion proteins, Cre-GRdex might be useful for achieving loxP site-directed mutagenesis in cultured cells and spatio-temporally controlled somatic cell mutagenesis in transgenic mice.

INTRODUCTION

Homologous recombination in embryonic stem (ES) cells is used as a routine technique for site-specific mutagenesis in mice (1). However, this approach has inherent limitations such as possible modification of the mutant phenotype due to the presence of the selection marker in the targeted gene or early mutant lethality (e.g. during embryonic development), which obviously preclude the analysis of the functions of the gene at later stages. A second severe limitation of this approach is that it does not allow modification of a gene in a spatio-temporally controlled manner (2). The Cre/LoxP system was developed to overcome these limitations. The Cre gene of bacteriophage P1 encodes a 38 kDa site-specific recombinase of the integrase family which efficiently promotes recombination at cognate-specific 34 bp sequences, termed loxP sites, in the absence of any additional cofactor. The fact that the Cre recombinase is active not only in bacteria, but also in yeast and higher eukaryotes, makes it a useful tool for further manipulations of the mouse genome (3).

We, and others, have shown that the activity of the Cre recombinase can be induced by ligands, when it is fused to the ligand binding domain (LBD) of nuclear receptors (4–6). To avoid the interference of endogenous ligands present in mice, Cre was fused to a human oestrogen receptor (ER) LBD containing point mutations known to impair oestradiol, but not tamoxifen (a synthetic ER antagonist) binding. Administration of tamoxifen to transgenic mice expressing this fusion protein was shown to induce loxP site-directed recombination in a number of tissues, whereas no excision could be detected in untreated animals (7,8), indicating that such chimeric Cre recombinase molecules had great potential for specific manipulations of the mouse genome.

We have recently identified a point mutation in the LBD of the human glucocorticoid receptor (GR) [I747→T; GR(I747T)] which alters its ligand specificity for transactivation (9). GR(I747T) is activated by synthetic ligands such as dexamethasone (dex), but not by natural GR ligands, even at high concentrations. We describe here a chimeric protein, Cre-GRdex, consisting of Cre linked to the LBD of GR(I747T) (Cre-GRdex), and analyze the recombinase activity of this chimeric protein in F9 murine embryonal carcinoma cells carrying a chromosomally-integrated loxP-flanked (‘floxed’) gene. We show that the synthetic GR ligands dex, triamcinolone acetonide (TA) and RU4846 (RU486) efficiently induce Cre-GRdex recombinase activity, whereas no activity is observed in the absence of ligand or in the presence of the natural GR ligands such as cortisol, corticosterone and aldosterone. Furthermore, as dex activates Cre-GRdex even in the presence of physiological concentrations of such natural ligands, this chimeric Cre recombinase should be useful for loxP site-directed spatio-temporally controlled somatic cell mutagenesis in mice.

MATERIALS AND METHODS

Expression vectors and cell lines

The expression vector pCre-GRdex was constructed by first inserting the T4 DNA polymerase-treated 1.3 kb Clal–XbaI DNA fragment of the GR(I747T) expression vector (9), encoding a human GR in which the isoleucine 747 was mutated to a threonine, into the T4 DNA polymerase-treated Xhol–PstI-digested expression vector pCre-ER (4). Subsequently, the Cre gene [amino acids (aa) 1–343 of orf2(Cre) (10)], the GR(I747T) LBD-encoding sequence [aa 500–777 (9,11)] and the sequence encoding the human ER F region [aa 553–595 (12)] were fused in frame by site-directed
mutagenesis using the oligonucleotides 5′-CTGGAAGATGTCGATCACTTGCTATCCGCAAGCCTA-3′ and 5′-CTGTTTCTC-TCAAAAGGGTACCCAGCTGAGGGGCACT-3′.

The RXRα+/–(LNL), RXRα+/–(LNL) and RXRα+/–(LNL) F9 murine embryonal carcinoma cell lines were cultured and electroporated as described (4,13). For transient transfections, 5 μg of supercoiled plasmid DNA was used. To establish stable lines constitutively expressing Cre-GRdex, cells were electroporated with 5 μg of SalI-digested pCre-GRdex and 1 μg of PvuII-linearized pPGK-hyg (14). Hygromycin resistant clones were obtained and amplified as described (4).

Detection of recombination events

Polymerase chain reactions (PCR) were performed as described (15), using the primers 5′-GGCAACACTGATG-3′ (SB211) and 5′-TTGCCGTCAGTGTTCTT-3′ (PZ105) (Fig. 1b). After denaturation for 8 min at 94°C, 2.5 U of Taq polymerase were added, and amplification was performed [36 cycles (30 s at 94°C, 30 s at 50°C) and 5 min at 72°C]. PCR products were separated either on a 10% polyacrylamide gel or on a 2% agarose gel and visualized respectively under UV after ethidium bromide staining, or blotted and hybridized to a γ-32P-radio-labelled oligonucleotide (5′-AATTATAACTTGCTAATAATGTACGTTACTAGGAATTTACCTA-3′;loxP site), as described (16).

Semi-quantitative PCR was used to estimate the relative level of DNA excision. The intensities of the signal corresponding to the PCR-amplified wild-type (+) and recombined [–(L)] DNA fragments separated on a polyacrylamide gel were determined by laser densitometry of Polaroid photographs of ethidium bromide-stained gels, after subtracting background staining. The recombination efficiencies were calculated as the ratio between the intensity of [–(L)] and (+) DNA fragments for RXRα+/–(LNL) treated cells, expressed as a percentage of the [–(L)]/(+) ratio obtained for the genetically characterized RXRα+/–(LNL) cell line (4).

RESULTS

Ligand-dependent recombinase activity of Cre-GRdex

To express a conditional Cre recombinase in which activity can be controlled by synthetic, but not by natural GR ligands, we constructed a pSG5-based (17) expression vector pCre-GRdex encoding a fusion protein consisting of Cre fused to the LBD of the human GR(747T) and tagged with the human oestrogen receptor (hER) F region (Cre-GRdex; Fig. 1a). Western blot analysis of pCre-GRdex transfected COS-1 cells extracts with an antibody directed against the F region of hER (18) showed that the chimeric protein was synthesized. Furthermore, immunofluorescence analysis of transiently transfected COS-1 cells revealed that Cre-GRdex was predominantly cytoplasmic in the absence of ligand, whereas it was nuclear when the cells were treated for 1 h with 10−6 M dex (data not shown). Thus, as previously shown for the tamoxifen-dependent Cre-ER fusion protein (8), ligand treatment is required for Cre-GRdex nuclear translocation.

The recombinase activity of Cre-GRdex was tested in the genetically modified F9 murine embryonal carcinoma cell line RXRα+/–(LNL), which contains a loxP-flanked neomycin resistance gene (LNL) inserted in exon 4 of the RXRα gene [Fig 1b: (4)]. RXRα+/–(LNL) cells were transiently transfected with pCre-GRdex or with the parental control vector pSG5, grown for 2 days in the absence or presence of 10−6 M cortisol or dex, and Cre-mediated excision was assayed. PCR amplification of wild-type (+) and recombined [–(L)] alleles with the primers SB211 and PZ105 used to detect the different alleles of RXRα+/–(LNL) transfected cells before [(+) and –(LNL)] and after [(+) and –(L)] excision, as well as the expected size of the amplified fragments, are indicated. (d) Detection of Cre-GRdex recombinase activity in transiently transfected RXRα+/–(LNL) F9 cells. Polyacrylamide gel analysis of the PCR fragments amplified from RXRα+/–(LNL) cells transiently transfected with either pCre-GRdex or the parental vector pSG5. Cells were treated for 2 days with vehicle alone or with either cortisol or dex at 10−6 M, as indicated. (+) and –(LNL) designate the PCR-amplified DNA fragment of RXRα+/–(LNL) wild-type and recombined alleles, respectively. M, HindIII-digested pBR322. (e) Detection of recombinase activity in RXRα+/–(LNL) F9 cells constitutively expressing Cre-GRdex. Polyacrylamide gel analysis of the PCR fragments amplified from genetically characterized F9 cells (lanes 1–4; (d)) and from RXRα+/–(LNL)Cre-GRdex cells treated for 3 days with vehicle alone or with either cortisol or dex at 10−6 M (lanes 5–7). (+) and –(LNL) designate the PCR-amplified DNA fragments of RXRα+/–(LNL) wild-type and recombined alleles, respectively. M, 1 kb DNA ladder (Gibco-BRL).

Figure 1. Recombinase activity of the chimeric Cre-GRdex protein. (a) Schematic representation of Cre-GRdex. The hatched, open and grey boxes represent the recombinase, the human GR(747T) LBD mutant and the hER F region, respectively. The upper numbers refer to amino acid positions in the chimeric protein; those in brackets correspond to positions in the parental proteins. (b) Schematic representation of wild-type (+), mutant [–(LNL)] and recombined [–(LNL)] alleles of RXRα+/–(LNL) F9 cells (4). The PCR primers (SB211 and PZ105) used to detect the different alleles of RXRα+/–(LNL) transfected cells before [(+) and –(LNL)] and after [(+) and –(L)] excision, as well as the expected size of the amplified fragments, are indicated. (c) Detection of Cre-GRdex recombinase activity in transiently transfected RXRα+/–(LNL) F9 cells. Polyacrylamide gel analysis of the PCR fragments amplified from RXRα+/–(LNL) cells transiently transfected with either pCre-GRdex or the parental vector pSG5. Cells were treated for 2 days with vehicle alone or with either cortisol or dex at 10−6 M, as indicated. (+) and –(LNL) designate the PCR-amplified DNA fragment of RXRα+/–(LNL) wild-type and recombined alleles, respectively. M, HindIII-digested pBR322. (d) Detection of recombinase activity in RXRα+/–(LNL) F9 cells constitutively expressing Cre-GRdex. Polyacrylamide gel analysis of the PCR fragments amplified from genetically characterized F9 cells (lanes 1–4; (d)) and from RXRα+/–(LNL)Cre-GRdex cells treated for 3 days with vehicle alone or with either cortisol or dex at 10−6 M (lanes 5–7). (+) and –(LNL) designate the PCR-amplified DNA fragments of RXRα+/–(LNL) wild-type and recombined alleles, respectively. M, 1 kb DNA ladder (Gibco-BRL).
To further evaluate the recombinase activity of this chimeric protein, RXRα+/-(LNL) F9 cells constitutively expressing Cre-GRdex were established. RXRα+/-(LNL) cells were co-electroporated with pCre-GRdex and a hygromycin resistance gene (pPGK-hyg); out of 48 hygromycin-resistant clones, 16 exhibited dex-dependent site-specific recombination, as determined using the above PCR strategy, indicating that these clones had efficiently integrated the Cre-GRdex expression cassette (data not shown). One of these clones, called RXRα+/-(LNL):Cre-GRdex, was treated for 3 days with vehicle alone or with 10^-6 M of either cortisol or dex, and recombination occurrence was revealed by PCR (Fig. 1d). Untreated or cortisol-treated RXRα+/-(LNL):Cre-GRdex cells yielded a single 65 bp fragment, as did the parental RXRα+/-(LNL) line (Fig. 1d, compare lanes 5 and 6 with lane 2). In contrast, treatment of these cells with dex led to the amplification of both 65 and 191 bp DNA fragments, a pattern similar to the one observed in RXRα+/-(LNL) cells (Fig. 1d, compare lanes 3 and 7). The relative intensities of the two fragments indicated that a 3 day 10^-6 M dex treatment resulted in >70% recombination.

To determine the kinetics of the dex-induced excision of the loxP-flanked DNA sequences, RXRα+/-(LNL):Cre-GRdex cells were treated with 10^-6 M dex for 0–120 h (Fig. 2). No recombination could be detected in the absence of dex. In contrast, the estimated recombination was ∼50 and 60% after 24 and 48 h treatments, respectively, and >80% after 120 h.

Cre-GRdex recombinase activity is induced by a variety of synthetic, but not by natural ligands of GR

To evaluate the recombinase activity of Cre-GRdex in the presence of natural (aldosterone, cortisol and corticosterone) or synthetic (dex, TA and RU486) GR ligands, RXRα+/-(LNL):Cre-GRdex cells were treated with these compounds for 2 days at concentrations ranging from 10^-9 to 10^-6 M, and recombination assayed as above (Fig. 3).

None of the natural GR ligands could activate the Cre-GRdex recombinase activity (Fig. 3, lanes 1–3, 7–9, 13–15 and 19–21), even at 10^-6 M. In contrast, at this concentration, all three

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**Figure 2.** Kinetics of Cre-GRdex recombinase activity in the presence of dexamethasone. (Upper) Polyacrylamide gel analysis of the PCR fragments amplified from the indicated cells and from RXRα+/-(LNL):Cre-GRdex cells treated with 10^-6 M dex for the indicated time. (+) and (−) are as in the legend of Figure 1d, and molecular sizes of Hinfl-generated pBR322 fragments are indicated on the right. (Lower) Levels of recombination were determined from RXRα+/-(LNL):Cre-GRdex cells treated with 10^-6 M dex for the indicated time. Circles, squares, triangles and crosses represent mean values of two PCR reactions from independent experiments. The results were expressed relative to the ratio of the intensity of the (−)/(+)-fragments obtained from the genetically characterized RXRα+/-(LNL) cells, taken as 100 (Materials and Methods).

**Figure 3.** Recombinase activity of Cre-GRdex in the presence of various concentrations of natural and synthetic GR ligands. RXRα+/-(LNL):Cre-GRdex cells were treated for 3 days with increasing concentrations of natural (aldosterone, cortisol and corticosterone) or synthetic (dex, TA and RU486) GR ligands. The recombinase activity was quantified by Southern blotting of PCR-amplified fragments separated on a 2% agarose gel, using a radio-labelled loxP-site oligonucleotide as a probe. The results were expressed relative to the activity measured in the presence of 10^-6 M TA (lane 23), taken as 100.
synthetic ligands induced a high recombination rate (Fig. 3, lanes 22–24). Interestingly, RU486, an anti-glucocorticoid exhibiting a partial agonistic activity (19,20), was ∼60% as efficient as TA at inducing Cre-GR<sup>dex</sup> recombinase activity when tested at 10<sup>−6</sup> M, while it was as potent as TA at 10<sup>−7</sup> M (Fig. 3, compare lanes 23 and 24, and 17 and 18, respectively). Furthermore, at 10<sup>−8</sup> M, RU486 was five times more efficient than TA, and at 10<sup>−9</sup> M only RU486 induced some recombination (Fig. 3, compare lanes 11 and 12, and 1–6, respectively). At any given concentration >10<sup>−8</sup> M, TA was always more potent than dex in activating Cre-GR<sup>dex</sup> recombinase activity (Fig. 3, compare lanes 10 and 11, 16 and 17, and 22 and 23).

**Cre-GR<sup>dex</sup> stimulation by dexamethasone in the presence of natural ligands of GR**

To investigate whether the presence of natural GR ligands would interfere with Cre-GR<sup>dex</sup> activation by synthetic ligands, we treated RXRα<sup>+/–(LNL)</sup>:Cre-GR<sup>dex</sup> cells for 3 days with dex in the presence of a mixture of the three natural GR ligands cortisol, corticosterone and aldosterone (Fig. 4). As expected, no recombination was observed in the absence of synthetic ligand, even in the presence of the natural ligands at a concentration of 10<sup>−6</sup> M (Fig. 4, lanes 1, 6 and 11). However, addition of 10<sup>−7</sup>–10<sup>−6</sup> M of dex to the medium induced the recombinase activity of the fusion protein, and the presence of either 10<sup>−8</sup> or 10<sup>−6</sup> M of natural ligands had almost no effect (Fig. 4, lanes 4 and 5, 9 and 10, and 14 and 15). Note that 10<sup>−6</sup> M dex brought a higher recombinase activity than 10<sup>−7</sup> M dex irrespective of the presence of natural ligands (Fig. 4, compare lanes 4 and 5, 9 and 10, 14 and 15, and data not shown), in agreement with the above data (Fig. 3).

**DISCUSSION**

The fusion of site-specific recombinases to ligand binding domains of steroid receptors generates ligand-dependent site-specific recombinases (4–6,21). However, the presence of steroids in mice precludes the use of wild type LBDs to tightly regulate the recombinase activity in transgenic animals. We have recently shown that a fusion between Cre and a mutated LBD of the human oestrogen receptor (Cre-ER<sup>T</sup>), which binds tamoxifen, but not oestradiol, generates a functional tamoxifen-dependent recombinase in transgenic mice (7,8). To obtain an additional ligand-inducible Cre recombinase insensitive to endogenous ligands, we have fused Cre to a mutated hGR LBD (Cre-GR<sup>dex</sup>). Indeed, the hGR<sup>(I747T)</sup> mutant which is transcriptionally inactive at physiological concentrations of natural GR ligands (10<sup>−8</sup>–10<sup>−7</sup> M), is active in the presence of synthetic ligands such as dex or TA (9).

Cre-GR<sup>dex</sup> has no activity in untreated or cortisol-treated transfected mouse embryonal carcinoma F9 cells, whereas a 2-day dex treatment efficiently induced site-specific recombination. To further characterize the Cre-GR<sup>dex</sup> recombinase activity, genetically modified F9 cells containing a ‘floxed’ gene and constitutively expressing the fusion protein were exposed to various concentrations of natural or synthetic GR ligands. As expected, physiological concentrations (10<sup>−8</sup>–10<sup>−7</sup> M) of the natural GR ligands aldosterone, corticosterone or cortisol did not activate Cre-GR<sup>dex</sup>, whereas dex, TA and RU486 effectively induced the recombinase activity. At high ligand concentration (10<sup>−6</sup> M), Cre-GR<sup>dex</sup> recombinase activity was similar in the presence of dex or TA, whereas RU486 was ∼60% as active. In contrast, at lower concentration, RU486 was either as efficient or more efficient than the two other synthetic compounds. These results are in good agreement with the relative efficiencies of dex and TA at inducing the transcriptional activity of GR(I747T) (9). Interestingly, although RU486 efficiently inhibited transactivation by GR(I747T) (9), it was a potent inducer of Cre-GR<sup>dex</sup> recombinase activity.

Kinetic studies of Cre-GR<sup>dex</sup> recombinase activity in the presence of dex revealed a high excision rate after 2 and 5 days of treatment (∼60 and 80%, respectively). Thus, Cre-GR<sup>dex</sup> appears to be as efficient as the previously described Cre fusion proteins (5,6,22) in achieving ligand-induced site-specific recombination.

The presence of natural GR ligands such as cortisol, corticosterone and aldosterone at physiological concentrations in the medium did not alter the activation of Cre-GR<sup>dex</sup> by dex. As both the Cre-GR<sup>dex</sup> and the previously reported Cre-ER<sup>T</sup> have no detectable background activity in the absence of ligand and can be activated by different synthetic ligands, they should be useful to generate independent spatio-temporally controlled site-directed somatic mutations in mice.

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