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Rapid report

Nuclear immobilization of DsRed1 tagged proteins: A novel tool for studying DNA–protein interactions?

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Abstract

DsRed1 is a red fluorescent protein that can be used as a fusion partner with other proteins to determine their subcellular localization, similarly to the popular green fluorescent proteins (GFP). Here, we report that fusion of DsRed1 to estrogen receptor α (ERα) renders the transcription factor immobile within the nucleus. Furthermore, we show that the immobilization is dependent on DNA interaction and that the binding to the DNA can be direct as well as indirect for DsRed to immobilize with its fusion partners. This observation could provide a new tool to be used for the identification of target genes containing low affinity binding sites for several transcription factors including ERα. In addition, it could be employed for studies on protein–DNA interactions as well as protein–protein interactions during protein complex formation on chromatin in the event of transcription initiation and regulation.

Keywords: Estrogen receptor; DNA interactions; DsRed; Nuclear protein mobility; Chromatin

Some drawbacks of the DsRed1 protein have been reported previously, namely its tendency to form aggregates as it has been shown to exist as an obligate tetramer both in purified form as well as in cells and it is further hampered by the slow maturation rate of the chromophore, going through GFP-like intermediates and reaching its maximum red fluorescent form only after 48 h [1–3]. The tetramerization was further confirmed in detail, 2 Å resolution structure analysis of the protein revealing a structure very similar to GFP and an almost identical chromophore although the similarity of the two proteins is only 22% [4,5]. In addition, the tetramerization has been proposed to be essential for the red fluorescence emitted by DsRed1 as monomers are devoid of fluorescence capability [6].

We have previously shown that a novel splicing variant of thioredoxin reductase 1 (TrxR1b) can bind to the estrogen receptors (ERα and ERβ) in the presence of estradiol (E_2) and translocate and colocalize with them in the nucleus [7]. In this process, while monitoring fluorescent recovery after photobleaching (FRAP) of cells co-transfected with ERα/β-GFP and TrxR1b–DsRed1, we discovered that while the ERs were mobile, as previously reported [8], surprisingly, TrxR1b was immobile (Fig. 1a). This suggested that perhaps TrxR1b was incorporated into the chromatin or it might have a structural function in the nucleus that caused the immobilization. However, repeating the same experiment using TrxR1b-HcRed [7] or with ERα/β-CFP and TrxR1b-YFP (Fig. 1b), we showed that TrxR1b was actually mobile; hence the immobility of TrxR1b-DsRed was most likely caused by the presence of DsRed1. In the presence of the antiestrogen ICI the ERs do not co-localize in the nucleus with the TrxR1b suggesting that under ICI treatment TrxR1b cannot bind to the ERs [7]. To test whether binding to ERs is needed for TrxR1b to become immobile, we co-transfected the ERα–GFP and TrxR1b–DsRed1 in the presence of ICI. Indeed, in the presence of ICI, ERα was immobile, as has previously been described [8], while the nuclear portion of TrxR1b was totally bleached suggesting that interaction of TrxR1b–DsRed1 with ER it necessary for immobilization (Fig. 1c).

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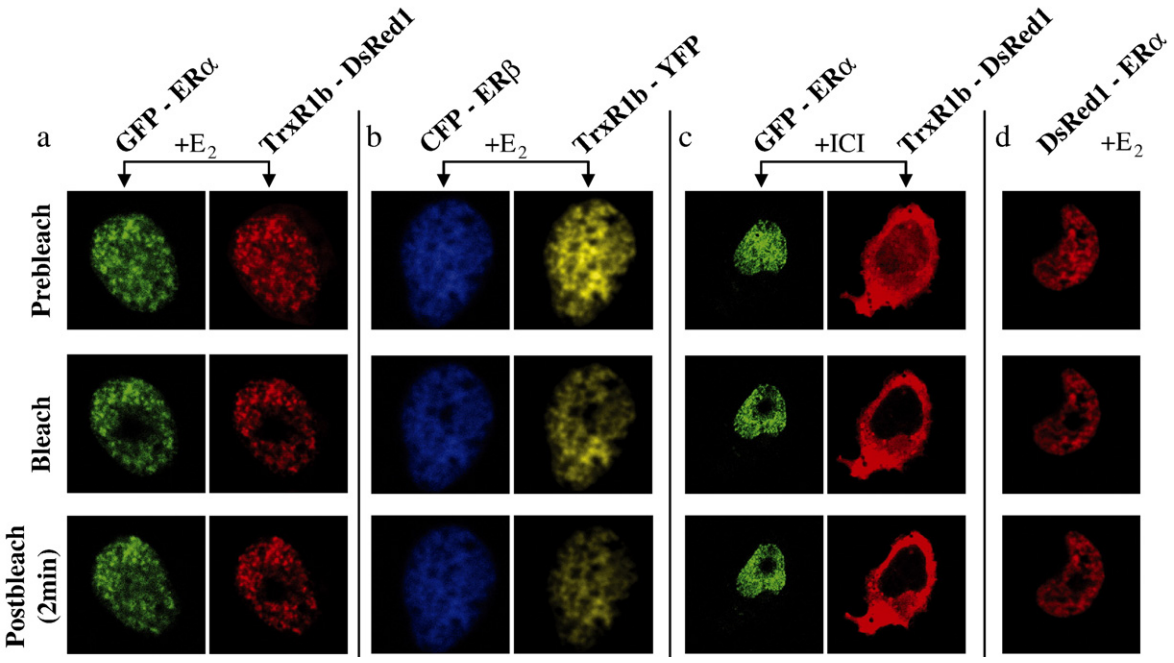

Fig. 1. (a–d) HEK293 cells were transfected with the indicated constructs using lipofectamin 2000 and treated with the indicated ligands (25 nM E_2 or 100 nM ICI). FRAP was performed by spot bleaching and subsequent time laps image acquisition. Experiments were carried out more than three times. A representative cell is shown.

Since TrxR1b–DsRed1 is dependent on its interaction with ERα–GFP to become immobilized, we then tested whether the ERs, which directly bind to the DNA, could also become immobile when fused to the DsRed1. Accordingly, when ERα–DsRed1 was transfected in the presence of E_2 it became immobile (Fig. 1d). There was a substantial difference in mobility between the ERα–GFP and ERα–DsRed1 in co-transfected cells as shown by the quantitative FRAP (Fig. 2a). Since TrxR1b is immobilized only in the presence of both ERs and E_2, our hypothesis was that DNA interaction is needed for the immobilization. To test this hypothesis we used a deletion mutant of ERα, lacking the DNA binding domain, fused to DsRed1 (ΔERα–DsRed1). Indeed, when transfected the deletion mutant showed a very rapid recovery (Fig. 2b), even more rapid than ERα–GFP. In fact, the recovery was so fast that the time between the bleaching and the capture of the first image allowed for a significant recovery compared to ERα–GFP, resulting in a bleached image of only 60% compared to the 40% of ERα–GFP and reaching 100% recovery faster.

The absence of a DNA binding domain in the ΔERα–DsRed1 construct, coupled to its rapid movement suggests that DNA binding is crucial for the immobilization by DsRed1 and that DsRed1 by itself cannot cause the immobilization. Even more interesting is the observation that the binding to the DNA does not have to be direct, rather indirect binding via another protein might also slow down the mobility as indicated by TrxR1b–DsRed1 and ER-GFP transfections. Thus, co-transfection of mutant ΔERα–DsRed1 with wt ERα–GFP should lead to the immobilization of ΔERα–DsRed1 since those two proteins should dimerize and bind to DNA in the presence of E_2. As shown (Fig. 2c), in the presence of E_2 and wt ERα–GFP, ΔERα–DsRed1 colocalized with the wt ERα–GFP and became immobilized suggesting that even indirect binding to DNA can cause the immobilization of a DsRed1 tagged protein, which most probably is also the mechanism underlying the immobilization of TrxR1b–DsRed1 by ERs. Furthermore, the ERα–GFP tagged protein showed a considerably slower recovery (compare Fig. 2a and c). One explanation for this discrepancy could be the fact that DsRed exists as an obligate tetramer thus the fused ERα partners might interact within the same tetramer and quickly becomes immobilized as they can bind DNA. Thereby it is less likely to bind to the ERα–GFP molecules as the ERα–DsRed chimeras will be effectively excluded from the mobile/soluble fraction of the ERα–GFP. On the contrary, the ΔERα–DsRed is a fast moving protein with no capability of binding DNA and therefore is more likely to interact with ERα–GFP which then can bind DNA, become immobilized and in doing so it will essentially also immobilize the ERα–GFP partner. Since the ERa dimers are very stable, especially in the presense of ligands [9], the recovery observed will be more a representation of the disassociation from the dimerization partner rather than the inherit mobility of the protein.

As previously mentioned, the use of DsRed1 poses problems, such as tetramerization and slow maturation, that prevent it from being an ideal partner for GFP in double labeling experiments We therefore used the recently introduced version of DsRed (Clontech), which has 45 amino acid substitutions to produce a monomeric protein with fast maturation rate and similar spectra properties of the old DsRed, to explore the possibility of tetramerization being important for the observed immobility. Indeed, this version of the protein does not seem to cause any slow down in the mobility of ERα (Fig. 2d), rather it has similar recovery properties to ERα–GFP, strongly suggesting that tetramerization is of importance for the DsRed1 pro-

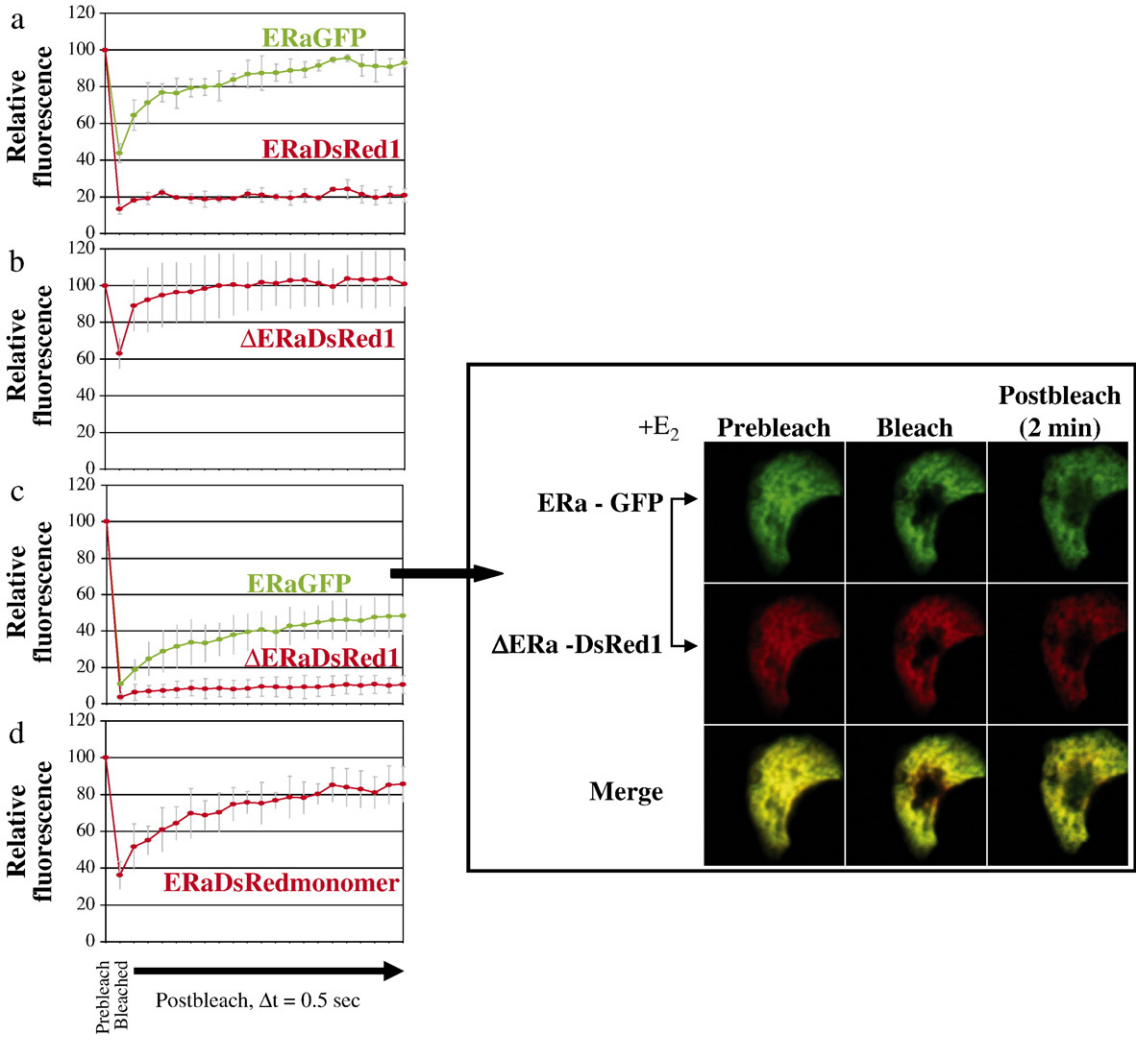

Fig. 2. (a–d) HEK293 cells were transfected with the indicated constructs and treated with 25 nM E_2. FRAP was performed on a Leica TCS SP2 confocal microscope. Cells were cultured on 35-mm diameter dishes and were kept on a 37 °C microscope stage during the imaging procedure. Cells were spot bleached for 2 s and the recovery was followed by time laps microscopy with a single scan 512×512 image captured every 0.5 s and imaged through a 40× dipping lens objective and. Shown are the average recovery curves as calculated from several cells. Bars indicate standard deviation. For panel c also a representative cell is shown.

perty to immobilize DNA binding proteins. Furthermore, the immobilized DsRed protein fusions are still emitting red fluorescence suggesting that they are in a tetramer form or at least dimmer form, as this is the minimum configuration in which the DsRed can produce red fluorescence [6].

We have here presented data showing that non DNA binding proteins through interaction with DNA binding proteins as well as DNA binding proteins themselves can become immobilized in the nucleus by DsRed1. As shown in Fig. 3a, transcription factors that can bind to DNA are dissociated at a constant rate rendering transcriptions factors highly mobile in the nucleus as has been shown for ERα [8]. Similar results have been reported for the glucocorticoid receptor [10] and the progesterone receptor B [11], showing quick cycling on their DNA binding sites. This has lead to the proposed "hit and run" mode of action for transcription factors and especially nuclear receptors [12–14]. However, fusing a transcription factor such as ERα with DsRed1 led to the immobilization of the protein in the nucleus (Fig. 3a). Similarly, proteins that do not interact with the DNA directly, are also dissociated at a constant rate leading to a mobile nature of the protein, but even in this case, fusion of the protein with DsRed1 results in immobilization (Fig. 3b). Although our data suggest caution in the interpretation of data using DsRed1, especially for studying mobility of nuclear proteins, more interesting is the potential to exploit this observation as a useful tool to study DNA–protein interactions.

Fusion of proteins to DsRed could be used to demonstrate that the fusion partner is a DNA binding protein *in vivo*. More specifically, we envision that fusion of DsRed to low affinity DNA binding proteins could lead to stabilization of their DNA binding to several target genes. It could also be used to demonstrate whether a certain treatment or condition of a cell can promote the DNA binding of a protein. Furthermore, it could potentially be used to study indirect protein binding to DNA or protein complex interaction or formation that can occur on the DNA during gene activation, suppression and transcrip-

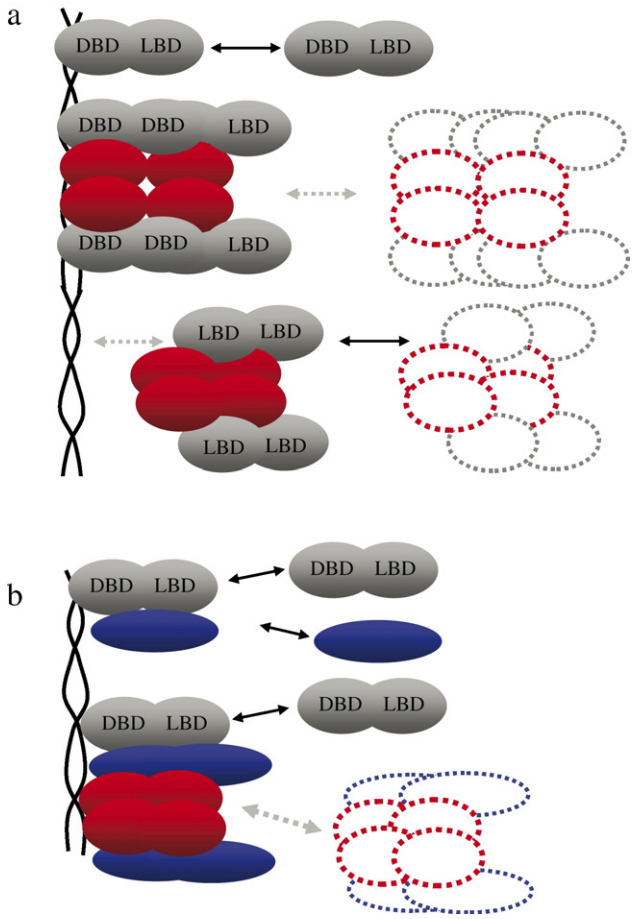

Fig. 3. Suggested model for the mode of action of DsRed1. (a) Transcription factors such as nuclear receptors associate with DNA at specific binding sites but the interactions are transient as the proteins are disassociated at a constant rate leading to the mobile nature of nuclear receptors (grey) in the nucleus. However, when fused to DsRed1 (red) the mobility of the protein is lost. In contrast, when the DNA binding domain (DBD) of the receptor is deleted the ligand binding domain (LBD) when fused to the DsRed is not capable to incur the immobilization observed by the full length protein. (b) Similarly to a, proteins (blue) that interact with other proteins on the DNA also show a transient nature in the interaction and can be highly mobile as well. When fused to the DsRed1 protein, although not capable of direct binding to DNA, proteins still become immobilized as also demonstrated with the deletion mutant ΔERα–DsRed1 which lacks the DNA binding domain but is still capable of immobilization in the presence of full length ERα.

tion. In addition, DsRed fusion proteins can be used to screen expression libraries for isolation of interacting partners that can exclusively bind DNA. Finally, libraries of short oligos fused and expressed with DsRed could be used in screening against a target GFP protein to isolate peptides that can bind to the target protein under certain conditions. One area where such a library could be of use is in the studies of nuclear receptors and coregulator interactions. Use of such a library could give a better understanding of how conformational changes induced by ligands promote or abolish interactions with certain type of peptides [15–17] when the receptors are bound to DNA, thereby giving a better understanding of interactions between nuclear receptors and coregulatory proteins in their natural environment. However, the molecular mechanism behind the observed phenomenon and its applicability to all proteins still remain to be clarified.

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