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RESEARCH ARTICLE

Nucleocytoplasmic shuttling of the glucocorticoid receptor is influenced by tetratricopeptide repeat-containing proteins

Gisela I. Mazaira[1,*], Pablo C. Echeverria[2,*] and Mario D. Galigniana[1,3,‡]

ABSTRACT

It has been demonstrated that tetratricopeptide-repeat (TPR) domain proteins regulate the subcellular localization of glucocorticoid receptor (GR). This study analyses the influence of the TPR domain of high molecular weight immunophilins in the retrograde transport and nuclear retention of GR. Overexpression of the TPR peptide prevented efficient nuclear accumulation of the GR by disrupting the formation of complexes with the dynein-associated immunophilin FKBP52 (also known as FKBP4), the adaptor transporter importin-β1 (KPNB1), the nuclear pore-associated glycoprotein Nup62 and nuclear matrix-associated structures. We also show that nuclear import of GR was impaired, whereas GR nuclear export was enhanced. Interestingly, the CRM1 (exportin-1) inhibitor leptomycin-B abolished the effects of TPR peptide overexpression, although the drug did not inhibit GR nuclear export itself. This indicates the existence of a TPR-domain-dependent mechanism for the export of nuclear proteins. The expression balance of those TPR domain proteins bound to the GR–Hsp90 complex may determine the subcellular localization and nucleocytoplasmic properties of the receptor, and thereby its pleiotropic biological properties in different tissues and cell types.

KEY WORDS: Glucocorticoid receptor, Tetratricopeptide repeats, Immunophilins, FKBP52, Hsp90, Nuclear matrix

INTRODUCTION

The glucocorticoid receptor (GR) is a ligand-activated transcription factor that belongs to the nuclear receptor superfamily. In the absence of hormone, GR is primarily located in the cytoplasm, whereas it rapidly migrates to the nucleus upon steroid binding (Pratt et al., 2004a). According to its nature as a ligand-activated transcription factor, the GR activates or represses the expression of cognate target genes. In its transcriptionally inactive form, the GR exists as an oligomeric heterocomplex with a dimer of the chaperone Hsp90, one molecule of Hsp70, the acidic co-chaperone p23 and a tetratricopeptide-repeat (TPR) domain factor, i.e. proteins containing sequences of 34 amino acids repeated in tandem (Galigniana et al., 2010a; Pratt et al., 2004b). This oligomeric structure is common for all members of the steroid receptor subfamily (Pratt and Toft, 1997; Smith and Toft, 2008). The high molecular weight immunophilins FKBP52 (also known as FKBP4), FKBP51 (FKBP5) and CyP40 (PPID), and the immunophilin-like proteins PP5, FKBPL (WISp39) and FKBP37 (ARA9/XAP2/AIP) are counted among the best characterized members of the TPR domain family of proteins associated with nuclear receptor family members via Hsp90 (Pratt et al., 2004a; Storer et al., 2011). TPR domains are composed of loosely conserved 34 amino acid sequence motifs that are found in arrays comprising between 2 and 20 repeats, wherein the repeats pack against each other giving rise to coiled and superhelical structures (Perez-Riba and Itzhaki, 2019). Originally identified in components of the anaphase-promoting complex (Sikorski et al., 1991), TPR domains are now known to mediate specific protein interactions in numerous cellular contexts. Usually, a short peptide of around 5 amino acids binds to the concave groove formed by the TPR solenoid over a span of 2–3 repeats, the highly negative charged MEEVD C-terminal end of the chaperone Hsp90 being the dominant interacting motif. A structural analysis of such interaction and its properties has recently been analysed in detail (Cauerhff and Galigniana, 2018).

The first structure of a TPR protein was determined for Hop/P60 (Scheufler et al., 2000), a co-chaperone responsible for the maturation cycle of steroid receptor heterocomplexes allowing the interaction of Hsp90 with Hsp70 (Pratt et al., 2004a,b). Nonetheless, Hop/P60 is not recovered in the final mature heterocomplexes of steroid receptors because it dissociates in intermediate steps leaving available the TPR-acceptor site of Hsp90 for binding of other TPR proteins, usually a high molecular weight immunophilin, which is the one that persists in the mature receptor–Hsp90 complex. These TPR domain proteins are implicated in various steps of the mechanism of action of the receptor, ranging from hormone binding and cytoplasmic retrograde transport to transcriptional regulation (Allan and Ratajczak, 2011; Pratt et al., 2004b; Ratajczak, 2015; Storer et al., 2011; Wochnik et al., 2005).

The Hsp90-associated TPR proteins play a key role in determining the subcellular localization of steroid receptors, in particular the high molecular weight immunophilins FKBP51 and FKBP52. Independently of their primary location, all steroid receptors are constantly shuttling in an active manner between nucleus and cytoplasm, such that the resulting equilibrium determines their final subcellular distribution in each particular physiological situation (Defranco et al., 1995; Mazaira et al., 2018; Shank and Paschal, 2005; Vandevyver et al., 2012). This property implies that soluble receptors must be transported towards the nucleus, a phenomenon that also includes those that are primarily nuclear (for example, the oestrogen receptor). Receptors are not statically confined within the nuclear compartment even when they show a mostly nuclear localization. Inasmuch as the cytoplasmic transport of the GR towards the nucleus is dependent on the Hsp90-based heterocomplex via dynein motors, the association with the chaperone oligomeric structure is a key requirement for the efficient nuclear accumulation of the receptor (Galigniana, 2012; Galigniana et al., 2010b, 2001). Importantly, the Hsp90-based trafficking machinery was verified by using other experimental models (Ebong et al., 2016; Harrell et al., 2002;

[1]Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires 1428, Argentina. [2]Département de Biologie Cellulaire, Université de Genève, Sciences III, Genève 1211, Switzerland. [3]Instituto de Biología y Medicina Experimental (IBYME)-CONICET, Buenos Aires 1428, Argentina.
*These authors contributed equally to this work

‡Author for correspondence (mgaligniana@conicet.gov.ar)

M.D.G., 0000-0002-9130-8574

Handling Editor: Maria Carmo-Fonseca
Received 4 September 2019; Accepted 7 April 2020

Journal of Cell Science (2020) 133, jcs238873. doi:10.1242/jcs.238873

McKeen et al., 2008; Tatro et al., 2009; Vandevyver et al., 2012; Wochnik et al., 2005) and has also been demonstrated for a great variety of soluble factors such as the catalytic subunit of telomerase (Jeong et al., 2016; Lagadari et al., 2016), the insect ecdysone receptor (Vafopoulou and Steel, 2012) or diphtheria toxin (Schuster et al., 2017), just to mention a few examples.

While the preferred TPR protein bound to Hsp90 in unliganded receptors is the immunophilin FKBP51, it is replaced by its homologous partner FKBP52 upon hormone binding (Davies et al., 2002; Gallo et al., 2007). Such immunophilin exchange in the early activation state of the receptor favours dynein recruitment (Harrell et al., 2002; Wochnik et al., 2005) and the initiation of the biological response. It has been proposed that conformational transitions in the β4–β5 loop of FKBP51 play a role in both the initial binding of FKBP51 to the unliganded steroid receptor as well as in the steroid-induced release of FKBP51 from the complex (Mustafi et al., 2014). Interestingly, overexpression of FKBP51 but not FKBP52 alters the subcellular localization of GR (Galigniana et al., 2010b). Regarding the capability of the TPR-domain immunophilin FKBP52 to bind the motor protein dynein, it appears to be redundant with other Hsp90-binding TPR proteins such as PP5, CyP40, FKBPL/WISp39 (Harrell et al., 2002; McKeen et al., 2008), but this property is not shown by FKBP51 (Wochnik et al., 2005).

Two nuclear localization signals (NLSs) have been characterized in the GR sequence (Picard and Yamamoto, 1987); one overlaps the C-terminal end of the DNA binding domain-hinge region (NLS1) and the other (NLS2) is located within the GR ligand-binding domain. The first step that favours the passage of the GR through the nuclear pore complex (NPC) involves the recognition of its NLS1 by the adaptor protein importin-α (Tanaka et al., 2003), followed by the formation of a trimeric complex with importin-β. Tethered within the NPC are several proteins called FG-nucleoporins (FG-Nups) that bear Phe-Gly (FG) repeat motifs (Aramburu and Lemke, 2017). These structures form a sieve-like hydrogel that can act as a barrier to repeal the passive diffusion of non-specific cargoes larger than 5 nm in diameter (~40 kDa) (Timney et al., 2016), such that the active canonical import pathway is usually favoured by the importin-α–importin-β1 complex. Nonetheless, an alternative importin-α-independent mechanism has also been proposed (Albermann et al., 2004; Piwien Pilipuk et al., 2007).

Glucocorticoids show pleiotropic biological actions ranging from neurodifferentiation and metabolic homeostasis to cell development and immune responses (Whirledge and DeFranco, 2018). Nonetheless, the number of factors able to regulate their actions is vast and variable among individuals, and our comprehension about how individual sensitivity *in vivo* to these hormones is regulated is not well understood. Despite the fact that it is largely accepted that the inactive GR is mostly cytoplasmic, such intracellular distribution is not always as clearly segregated as this model would suggest, with significant nuclear GR observed even under ligand-free conditions (Galigniana et al., 2010b) or during different steps of the cell cycle (Matthews et al., 2011). Obviously, dissecting the regulatory mechanisms for GR subcellular redistribution would be invaluable to aid understanding of the basic mechanisms that regulate glucocorticoid responsiveness in different tissues.

In view of the fact that the Hsp90–FKBP52–dynein complex associated to steroid receptors is essential for their cytoplasmic transport towards the nucleus (Galigniana et al., 2010a,b; Pratt et al., 2004a; Storer et al., 2011), one interesting extrapolation of this model is the possibility that the whole heterocomplex could interact directly with structures of the NPC. *In silico* analysis of the potential interactome of the GR–Hsp90 complex has suggested that various factors related to the nuclear import machinery could interact with the GR heterocomplex and consequently regulate its subcellular localization (Echeverria et al., 2011, 2009). Among them, there are proteins normally located in the perinuclear region such as importins, and others associated with the nuclear pore barrel, such as nucleoporins. All of these are candidates to form complexes with the receptor and associated chaperones. In a previous study we demonstrated that importin-β1 (KPNB1) and the nuclear pore glycoprotein Nup62 are chaperoned by Hsp90 (Echeverria et al., 2009), with these complexes reconstituted using a reticulocyte lysate system. Interestingly, these reconstitutions demonstrated that most of the other members associated with the steroid receptor heterocomplex such as Hsp70, p23, FKBP52 and PP5 were also recovered bound to Nup62, interactions that should facilitate GR nuclear import (Echeverria et al., 2009). Importantly, the experimental evidence indicates that the GR–Hsp90 complex translocates intact through the NPC, such that receptor transformation, i.e. the dissociation of the chaperone complex and subsequent GR dimerization, must both be nucleoplasmic events (Echeverria et al., 2009; Galigniana, 2012; Galigniana et al., 2010b; Grossmann et al., 2012; Presman et al., 2010). Since in a previous study (Galigniana et al., 2010b) it was shown that the GR localization is controlled by the recruitment of TPR domain proteins, and because preliminary experiments have shown that the overexpression of other TPR proteins than FKBP52 delocalizes nuclear GR from the nuclear compartment, we decided to analyse the actual interaction of the GR with those nuclear structures and the putative role of TPR domain proteins in the nuclear import–export mechanism of the receptor. Our study demonstrates a strong relationship between TPR proteins and the nuclear import mechanism of the receptor, as well as their potential capability to favour the anchorage of the GR to nuclear structures.

RESULTS

Interaction of GR with importin-β1 and Nup62

Based on the protein–protein interaction network predicted *in silico* for the GR–Hsp90 complex, we have previously reported that the nuclear pore glycoprotein Nup62 and the HEAT-domain repeat karyopherin importin-β1 are chaperoned by Hsp90 (Echeverria et al., 2009). These associations were shown by co-immunoprecipitation assays from L929 cell lysates and by heterocomplex reconstitution of the stripped immunoadsorbed protein with the reticulocyte lysate system as source of chaperones. In the present study, the interaction of the GR with both Nup62 and importin-β1 is shown in intact cells by confocal microscopy co-localization, which is in agreement with the previously demonstrated association between both proteins. Fig. 1 shows typical cytoplasmic scattered and nuclear surface punctuated staining pattern of importin-β1 (Bastos et al., 1997; Di Francesco et al., 2018; Fan et al., 2007) as well as the perinuclear staining pattern of Nup62, both colocalizing partially with the GR signal in some speckles. When cells treated with cortisol for 5 min (Fig. 1B) were compared with cells grown in a medium without steroid (Fig. 1A), it can be seen that GFP-GR has become more nuclear and also shows a greater partial co-localization with both Nup62 and importin-β1 at the nuclear envelope area. This is better visualized in the magnified areas shown on the right-hand side of the merged panel. After 20 min with cortisol, the shuttling equilibrium of the GFP-GR became nuclear in almost all cells. While the receptor was still showing co-staining with Nup62 at the nuclear periphery, the co-localization of GFP-GR with importin-β1 faded markedly to become undetectable. These observations in intact cells were confirmed by the observed pattern of chaperones and nuclear pore-related proteins after co-immunoprecipitation with the GR (Fig. 1D). Note the apparent

Journal of Cell Science (2020) 133, jcs238873. doi:10.1242/jcs.238873

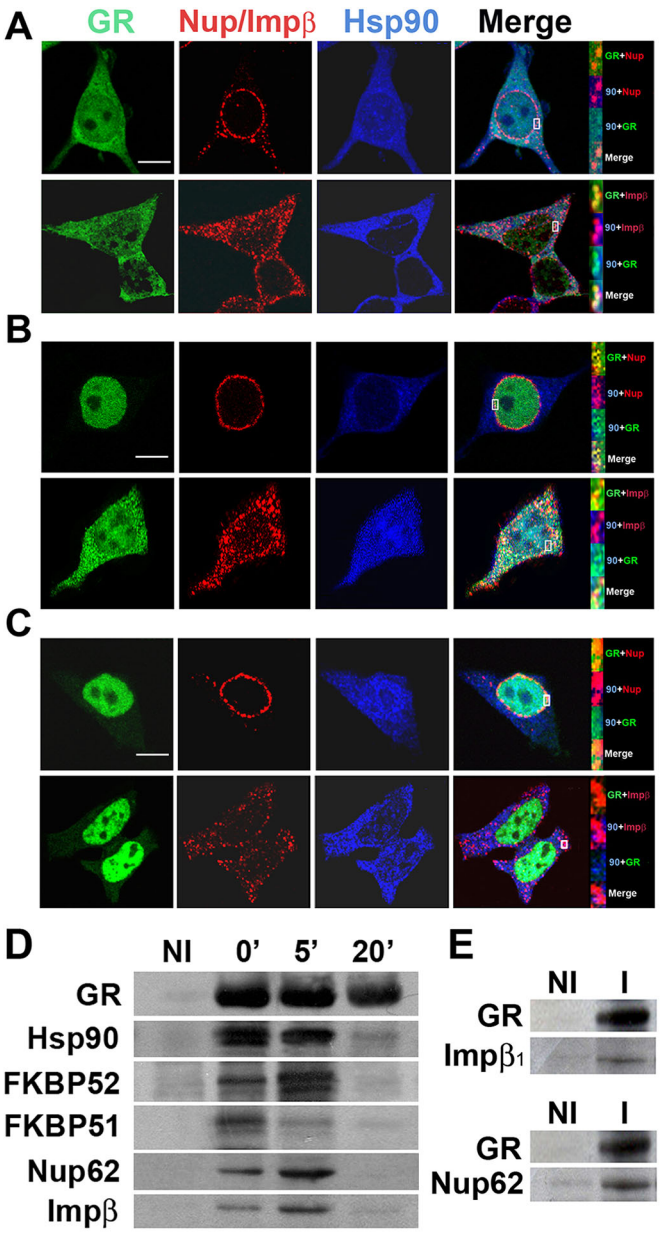

Fig. 1. GR interacts with importin-β1 and Nup62 in intact cells. GFP-GR-transfected 3T3-L1 mouse fibroblasts were grown on coverslips in a steroid-free medium. GR nuclear translocation was triggered by adding 10 nM cortisol. Coverslips were rinsed before adding the steroid (A) and after 5 min (B) or 20 min (C). Indirect immunofluorescence for Hsp90 (blue fluorescence) and Nup62 or Impβ1 (red fluorescence) was detected. Cells were visualized by confocal microscopy. The extreme right panel shows the magnified images of the white square on the nuclear envelope in the 'Merge' panel. (D) Pattern of proteins co-immunoprecipitated with the GR at the indicated incubation times with cortisol. (E) Co-immunoprecipitation of Impβ1 (top) and Nup62 (bottom) along with the GR in untreated cells grown in regular medium. NI, non-immune IgG; I, anti-GR IgG (BuGR2 monoclonal antibody). Scale bars: 10 µm.

increase of Nup62 and importin-β1 in the immunopellet after 5 min with steroid and the expected exchange of FKBP51 by FKBP52 in the GR–Hsp90 complex (Davies et al., 2002; Galigniana et al., 2010b; Gallo et al., 2007). After 20 min with steroid, only the chaperone-free GR could be immunoprecipitated after protein extraction from chromatin. Fig. 1E confirms that both nuclear pore-related proteins Nup62 and importin-β1, are capable to form complexes with the GR since they were co-immunoprecipitated with the receptor. Taken together, these observations strongly support the notion that the GR interacts physically with nuclear pore proteins in intact cells and suggest that these complexes should be transiently stable for a few minutes during the early steps of the GR nuclear accumulation process.

Impairment of GR nuclear accumulation by overexpression of a TPR peptide

The most frequent TPR domain proteins found associated with mature steroid receptor–Hsp90 complexes are PP5, CyP40, FKBP51 or FKBP52 (Erlejman et al., 2013). While CyP40 is usually not recovered with native GR complexes, but in reticulocyte lysate GR reconstituted systems (Galigniana et al., 1999; Owens-Grillo et al., 1995), it is more prevalent in PR and ER heterocomplexes (Barent et al., 1998; Gougelet et al., 2005; Ratajczak, 2015). On the other hand, FKBP51 is the TPR factor that shows preferential affinity for the Hsp90 acceptor site in unliganded steroid receptors and is replaced by FKBP52 (Davies et al., 2002) and/or PP5 (Gallo et al., 2007) upon steroid binding, thus favouring the nuclear accumulation of the receptor in a dynein-powered manner. In a previous study, we observed that the overexpression of FKBP51 impairs the nuclear localization of steroid receptors (Galigniana et al., 2010b), such that their nuclear accumulation was inversely proportional to the FKBP51:FKBP52 expression ratio. Because we have previously demonstrated that the TPR domain of the immunophilin is required for the interaction of these co-chaperones with nuclear pore structures (Galigniana et al., 2010b), we therefore assessed the potential influence of the TPR peptide in the nuclear accumulation mechanism of the GR.

HEK293T cells were co-transfected with EGFP-GR and pCMV6-Flag-TPR at a 10:1 ratio. Fig. 2A shows that after 20 min of cell stimulation with cortisol, the green fluorescence of the receptor is entirely nuclear in all cells (a quantification of the cytoplasmic and nuclear fluorescence is depicted as a bar graph in Fig. 2C). Notably, control cells overexpressing Flag-TPR (Fig. 2B, red-stained cells by indirect immunofluorescence with an anti-Flag IgG) already show a significantly low level of basal green fluorescence in their nuclei, whereas the addition of steroid to the medium resulted in an even lower accumulation of GFP-GR in the nuclear compartment. Thus, some residual fluorescence can still be seen in the cytoplasm after steroid stimulation. This indicates that the TPR peptide interferes with the nuclear accumulation process of the receptor, a fact that was confirmed by western blot analysis of cytosolic and nuclear cell preparations (Fig. 2D). To determine whether the nuclear pore factors shown in Fig. 1 associated with the GR heterocomplex are also affected by the overexpression of the TPR peptide, cells were placed on ice to permit the saturation of the receptor with cortisol without triggering its nuclear translocation, which is a temperature- and energy-dependent process (Gallo et al., 2007). Hence, the GR was immunoprecipitated and proteins were resolved by western blot (Fig. 2E). Both immunophilins were displaced by the TPR peptide, including FKBP52, an immunophilin usually recruited by the complex in a steroid-dependent manner. Accordingly, Nup62 and importin-β1 were dissociated, indicating that the main interaction in the GR heterocomplexes involves the immunophilin.

TPR overexpression delays GR passage through the NPC

In view of the results of Fig. 2, the nuclear accumulation of GFP-GR was measured along the exposure time to steroid. Fig. 3A shows that under regular culture conditions, GFP-GR exhibits the typical GR subcellular localization previously observed in the time-course monitoring after the addition of steroid, i.e. a rapid nuclear

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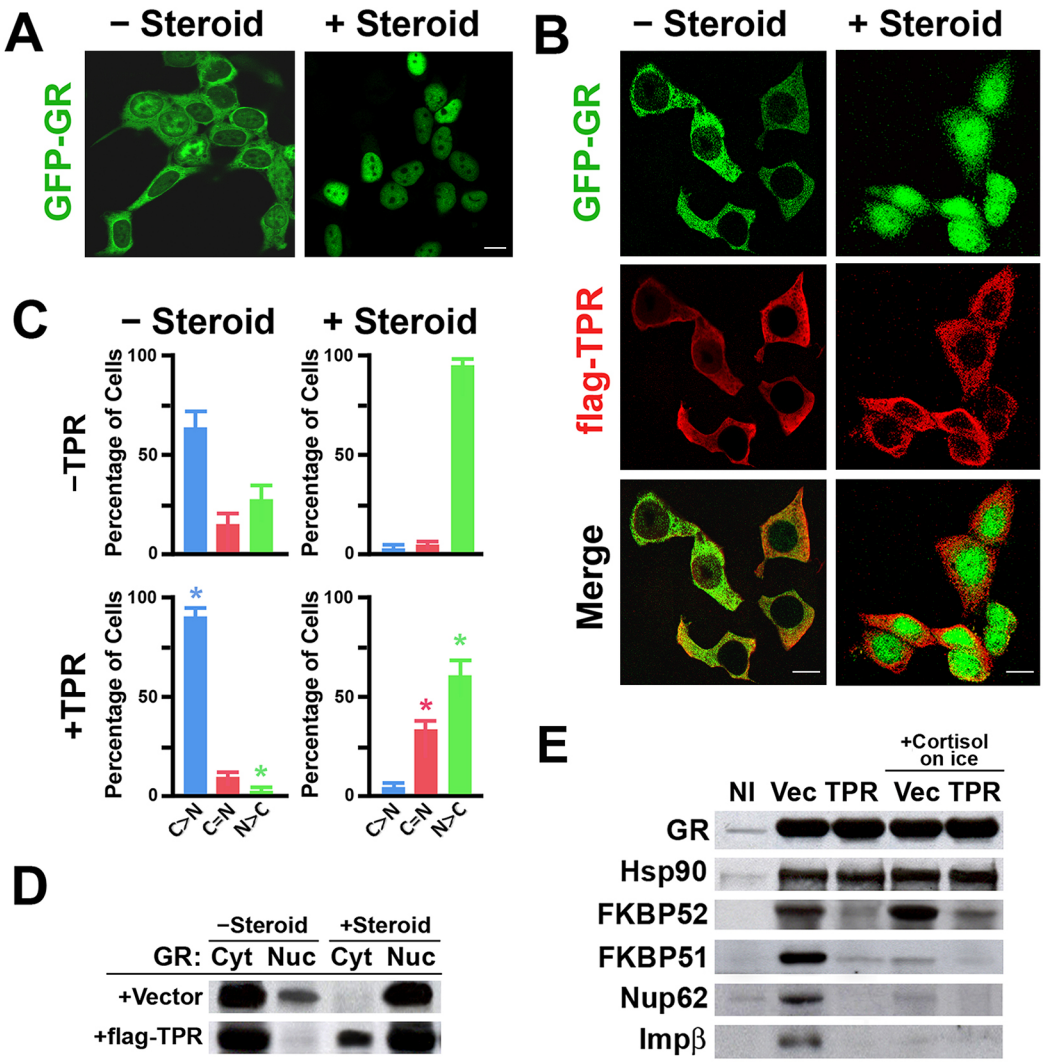

Fig. 2. Overexpression of the TPR peptide impairs GR nuclear accumulation. HEK293T cells grown in a steroid-free medium were co-transfected with EGFP-GR and pCMV6 empty vector (A) or EGFP-GR and pCMV6-Flag-TPR (B). The next day, cells were fixed after 20 min with 10 nM cortisol, stained with anti-Flag antibody for indirect immunofluorescence (red cells in B) and visualized by confocal microscopy. (C) Bar graphs depicting the quantification of pixels of GFP-GR green fluorescence for GFP-GR transfected cells (top) and Flag-TPR co-transfected cells (bottom), where C>N (blue) is mostly cytoplasmic fluorescence, N>C (green) is mostly nuclear fluorescence, and C=N (red) is an equally distributed fluorescence. **P*<0.05 when the Flag-TPR co-transfected cells shown in the lower bar graphs (+TPR) are compared with the corresponding condition of GFP-GR-transfected cells of the upper bar graph (–TPR). Results are the mean±s.e.m. for at least 100 cells per condition. (D) Subcellular fractionation of HEK293T cells co-transfected with pCMV6 empty vector or pCMV6-Flag-TPR showing the cytoplasmic (Cyt) and nuclear (Nuc) fractions of GR after 20 min with 10 nM cortisol (or vehicle). (E) The GR-bound heterocomplex was co-immunoprecipitated with the GR from extracts of cells co-transfected with empty vector (Vec) or Flag-TPR peptide (TPR). Cells were placed on ice and treated with 1 µM cortisol for 40 min to saturate the receptor with steroid (avoiding its nuclear translocation). The GR was immunoprecipitated and the associated proteins were resolved by western blot. NI, non-immune IgG. Scale bars: 10 µm.

accumulation that reaches a maximum after 15–20 min. Fig. 3B shows the quantification of the nuclear fluorescence. The overexpression of the TPR peptide largely delayed nuclear accumulation of the GR by almost an order of magnitude (half-life of ∼4–5 min versus ∼45 min).

Importantly, the GR movement to the nucleus was accompanied by a GFP-GR–nucleoporin association/disassociation recognizable pattern that varied along the experiment. It reaches a maximal co-localization 15–20 min after steroid addition, and then it fades to become almost residual after 80–90 min (see magnified inserts of the nuclear envelope area in Fig. 3A and the quantified co-localization index depicted in Fig. 3C). Such significant reduction in the co-localization index of the receptor with nuclear pore structures could be related to a lesser shuttling intensity across the nuclear pore. Fig. 3B demonstrates that the overexpression of the TPR-R101A mutant showed no significant differences with the control condition, consistent with its incapacity to interact with Hsp90 and the putative lack of effect in the active trafficking of the GR through the nuclear envelope. Because the TPR domain interacts with the MEEVD C-terminal sequence of Hsp90 (Cauerhff and Galigniana, 2018), the R101A mutation precludes the formation of regular hydrogen bonds between the R101 residue of the TPR domain and D5 and E5 amino acids of Hsp90, as shown by the molecular modelling scheme of Fig. 3D. To test the efficiency of the Flag-tagged TPR construct to disrupt Hsp90–TPR complexes, Hsp90 was immunoprecipitated from cell lysates overexpressing either TPR domain or TPR-R101A mutant and the co-immunoprecipitation of FKBP52 was visualized by western blot and compared to the control condition (Fig. 3E). As expected,

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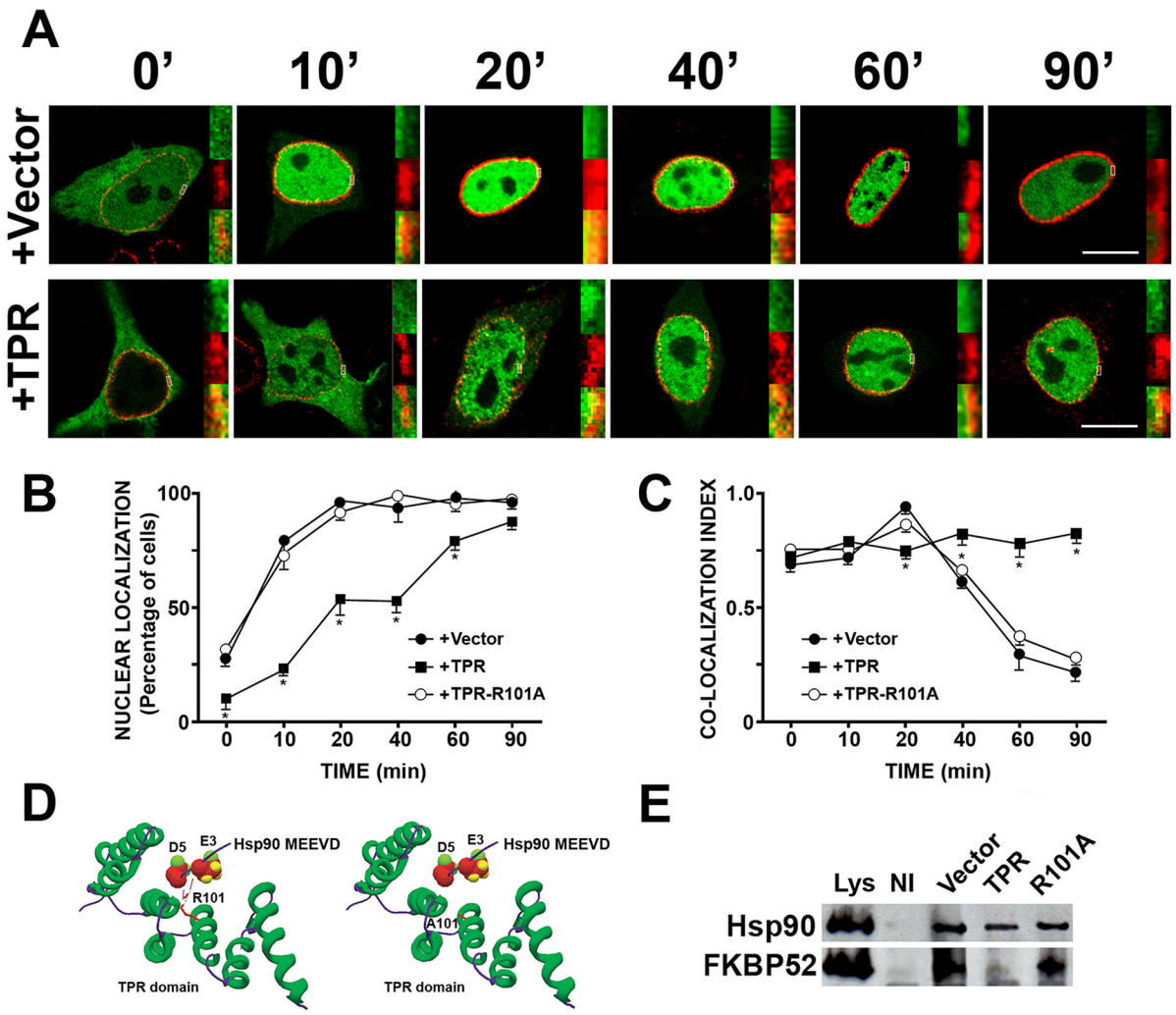

Fig. 3. TPR overexpression delays GR passage through the NPC. (A) HEK293T cells were co-transfected with EGFP-GR and either pCMV6 (+Vector) or pCMV6-Flag-TPR (+TPR) at a 10:1 ratio, and the GR nuclear translocation was triggered with 10 nM cortisol. The magnified insets show the GR (green)-Nup62 (red) co-localization for the regions indicated by white boxes. (B) The plot shows the nuclear accumulation of GFP-GR versus the incubation time with 10 nM cortisol. Results are the mean±s.e.m. for three independent experiments where more than 100 cells were scored in each one. (C) Co-localization score for images shown in the insets of A showing the colocalization of the receptor and Nup62. Data are mean±s.e.m. (D) Molecular modelling showing the lack of interaction of the R101A mutant of FKBP52 with the D5 and E3 residues of the MEEDV C-terminal sequence of Hsp90. (E) Co-immunoprecipitation of FKBP52 with Hsp90 from cell extracts overexpressing the TPR peptide or its R101A point mutant. NI: non-immune IgG. *P<0.05, compared with cells transfected with empty vector. Scale bars: 10 µm.

Hsp90 could be co-immunoprecipitated from wild-type and R101A mutant samples, but not from TPR-overexpressing lysate. Thus, the effect observed by the overexpression of the TPR peptide may be assigned to the disruption of Hsp90 from cognate associated TPR proteins. All these data suggest that when the GR–Hsp90–TPR heterocomplex is disrupted, there is a remarkable impairment in the nuclear accumulation of the receptor, an effect quite remarkable in the absence of hormone, but also noticeable under cortisol action.

Effect of leptomycin-B on GFP-GR shuttling

The reduced nuclear accumulation of the GR observed in TPR-peptide-overexpressing cells under both basal and steroid-dependent conditions may be the consequence of a less efficient nuclear import due to the disruption of the Hsp90–FKBP52–dynein transport machinery. However, an exacerbated nuclear export may also take place. To test the latter possibility, the CRM1 (exportin-1) inhibitor leptomycin-B was assayed. Cells grown on coverslips were exposed to steroid, the medium was washed by a steroid-free medium supplemented with the inhibitor, which was always present along the remaining time of the experiment. Fig. 4A shows that the CRM1-dependent pathway blocker has no significant effect in the nuclear accumulation of the GR. Strikingly, leptomycin-B prevents the inhibitory action observed by TPR overexpression. This was also noticeable when the GR–Nup62 co-localization index was measured (Fig. 4B). Because of these observations, a single cell was monitored along the incubation time with cortisol. Fig. 4C shows the fluorescence image of the selected cells for each condition and Fig. 4D depicts the individual measurements for nuclear fluorescence, which confirm that leptomycin-B abrogates the effect of the overexpression of the TPR peptide. This is particularly obvious during the first 12–15 min of GR export, after which the overall distribution of GFP-GR appears to converge into the nucleus. Taken together, these data suggest that there may exist a putative role of the GR export machinery that is evidenced only when the cognate TPR protein(s) are disrupted from GR–Hsp90 complexes. Furthermore, the effect of the TPR domain overexpression could be attributed to

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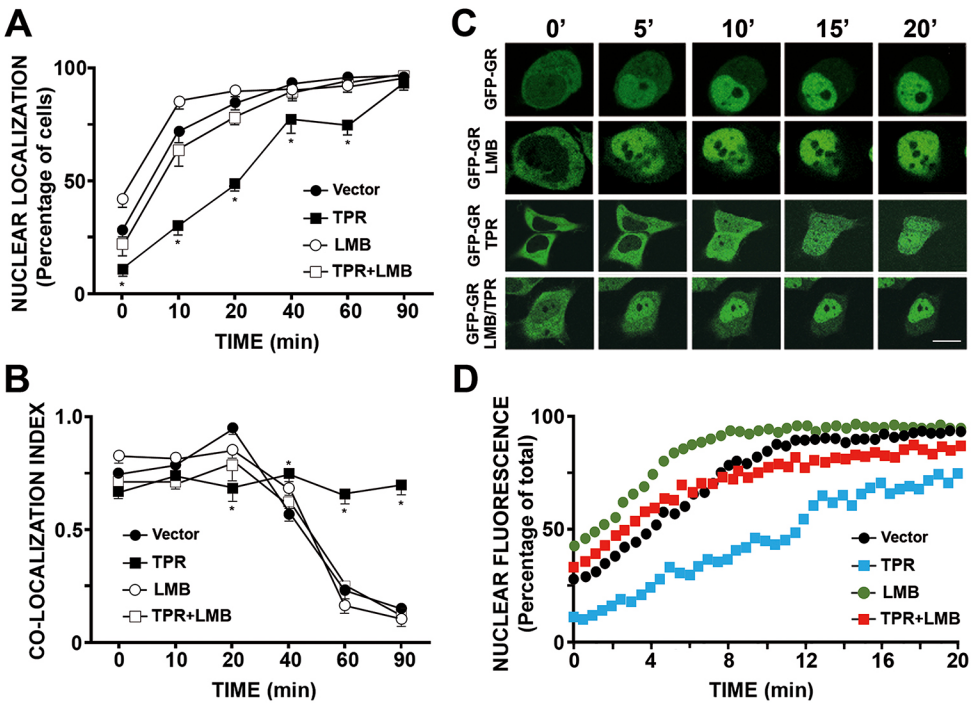

Fig. 4. Leptomycin-B inhibits the effect of TPR overexpression on GFP-GR shuttling. (A) The nuclear accumulation of GFP-GR was quantified upon the addition of 10 nM cortisol in HEK293T cells co-transfected with empty vector or the TPR peptide, in the presence or absence of 10 ng/ml lepromycin B (LMB). Results are the mean±s.e.m. for more than 100 cells. (B) Co-localization index for the NPC fluorescence was quantified as in Fig. 3C for the same conditions described for A. (C) Images of the nuclear translocation of GFP-GR recorded along the incubation time with cortisol for single cells. (D) Quantification of the nuclear pixels of GFP-GR for the cells shown in C. *P<0.05, compared with cells transfected with empty vector. Scale bar: 10 µm.

actions mostly related to export pathways, since the treatment with leptomycin-B restored control features by itself.

These observations led us to analyse the potential implication of CRM1 pathways in GR nuclear export. Cells were exposed to 10 nM cortisol for 1 h, the medium was replaced by a steroid-free medium, and cells were fixed a different incubation times in the absence of hormone. Fig. 5A shows that the overexpression of the TPR domain accelerated GR export profile. Surprisingly, while leptomycin-B did not show itself a significant effect on the regular GR export process (which should not consequently be related to the CRM1 pathway), it was also able to abolish the action of the TPR domain, such that the GR export rate was undistinguishable from the control condition. Inasmuch as this result was contradictory with the former and suggests that CRM1 could be involved, the expression of the protein was silenced with a specific siRNA (Fig. 5B). Knockdown of CRM1 did not affect either the GR nuclear export rate or the effect of leptomycin-B on the TPR-dependent GR export mechanism. This confirms that neither the regular export mechanism of the GR nor the inhibitory action of the TPR peptide on the GR export mechanism is related directly to the CRM1 export pathway. It should be noted that control experiments for the expected effect of leptomycin-B were also performed in parallel for the RelA/p65 subunit of the transcription factor NF-κB, a well-documented export system that uses CRM1 in a leptomycin-B-sensitive manner (Birbach et al., 2002; Ghosh et al., 2008; Kumar et al., 2004). As expected, treatment with leptomycin-B or the direct knockdown of CRM1 with specific siRNA fully retained RelA/p65 concentrated in the nucleus (Fig. S1).

To confirm the effect of the overexpression of the TPR peptide on the GR export mechanism by a different approach, a transient co-transfection with GFP-GR and Flag-TPR peptide followed by interspecies heterokaryon protein exchange was assayed. HEK293T human cells were co-transfected with EGFP-GR and pCMV6 or pCMV6-Flag-TPR. Then, these cells were fused with mouse L929 fibroblasts also transfected with pCMV6 or pCMV6-Flag-TPR to form heterokaryons after a shock with PEG. Cells were pretreated with cycloheximide, an antibiotic that was maintained along the entire experiment to avoid protein synthesis in the heterokaryons. At 2 h post-fusion, the cells were fixed and stained to control the expression of the Flag-tagged TPR peptide in heterokaryons by immunofluorescence (not shown). To distinguish human from mouse nuclei, cells were stained with DAPI to detect the characteristic intranuclear speckles shown only by mouse nuclei. In Fig. 5C, the visualization of the green fluorescence of GFP-GR (originally shown by human cells) in the mouse nuclei of the heterokaryons demonstrates GFP-GR shuttling, and therefore the nuclear export of the receptor from the human nuclei. Note that, in contrast to the fully nuclear localization of GR in control heterokaryons, those cells overexpressing the TPR peptide also show cytoplasmic signal, which may be the result of both an inefficient nuclear import and greater nuclear export.

FKBP52 favours GR anchorage to the nuclear matrix

The subcellular distribution of endogenous GR and FKBP52 was analysed by confocal microscopy in 3T3-L1 cells grown in the presence of cortisol because this cell type shows a prominent punctuated nuclear localization of the receptor. Fig. 6A shows that the nuclear speckle localization shown by both proteins in control cells is more diffuse in the TPR-overexpressing cells, suggesting that a peptide excess delocalizes both GR and FKBP52 from nuclear structures (see also the magnified nuclei shown at the bottom of Fig. 6B for those cells indicated by white arrows in A). It has been known for many years that in the nuclear compartment, steroid receptors are mostly attached to the nuclear matrix (Barrett and Spelsberg, 1999; DeFranco and Guerrero, 2000). Because indirect immunofluorescence images showed co-localization of the GR with FKBP52 in nuclear speckles (see Fig. 6A,B), the possibility that FKBP52 may be a nuclear matrix-associated factor was investigated. If this is the case, it would be entirely possible that the immunophilin could function as a potential anchorage nuclear factor for the GR via the TPR domain, such that the overexpression of this peptide would increase the population of 'free' (not attached)

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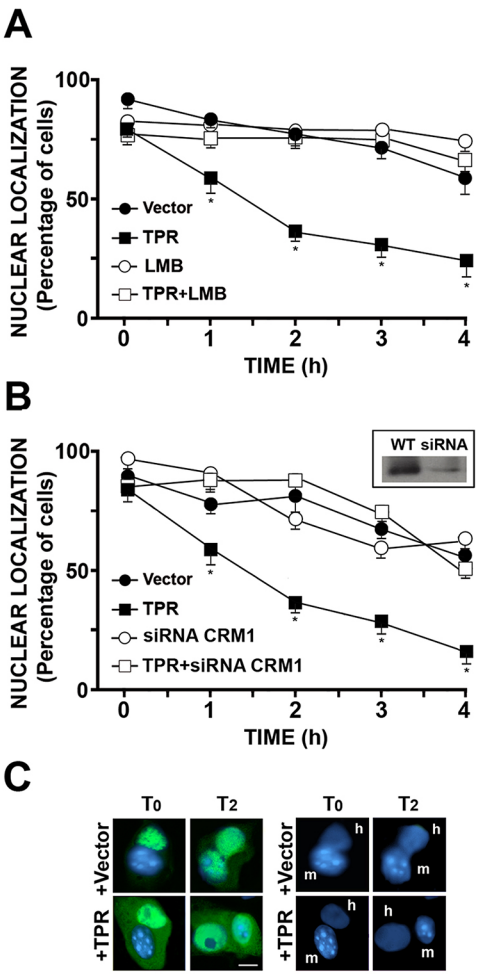

Fig. 5. Nuclear export of GFP-GR upon steroid withdrawal. (A) Cells co-transfected with empty vector or TPR peptide were incubated with 10 nM cortisol, washed and reincubated in a steroid-free medium in the absence or presence of 10 ng/ml leptomycin-B (LMB). Cells were fixed at different times and the remaining nuclear fluorescence was quantified. Results are the mean ±s.e.m. for more than 100 cells per condition. (B) CRM1 was knocked down with a specific siRNA. Then, the nuclear export of the GFP-GR was quantified as detailed in A. The inset shows the western blot for CRM1 in wild type (WT) and knockdown (siRNA) cell lysates. (C) Heterokaryons from HEK293T human cells expressing GFG-GR in the nucleus by preincubation with 10 nM cortisol and L929 mouse fibroblasts were generated using a PEG shock. Both cell types had been co-transfected with either empty pCMV6 vector or pCMV6-Flag-TPR. Immediately after the cell fusion was achieved (T_0), cells were reincubated in steroid-free medium supplemented with cycloheximide for 2 h (T_2), fixed and the green fluorescence observed by confocal microscopy. DAPI staining (blue nuclei, right panel) allows identification of human (h) and mouse (m) nuclei in the heterokaryons. *P<0.05, compared with cells transfected with empty vector. Scale bar: 10 µm.

nuclear GR thus favouring its nuclear export. Fig. 6B shows that the nucleoskeleton-associated pool of the GR dramatically decreases when the TPR peptide is overexpressed. Such an effect parallels the dissociation of FKBP52 (also suggested by the reduction of the speckle signal), suggesting a key role of the TPR domain in the anchorage mechanism of FKBP52 to the nuclear matrix and consequently the nuclear half-life of unliganded GR.

DISCUSSION

In previous studies, we demonstrated that TPR-domain immunophilins affect the subcellular localization of steroid receptors (Galigniana, 2012; Galigniana et al., 2010b, 2001; Mazaira et al., 2014). Thus, it was shown that FKBP52 is required for the nuclear accumulation of the receptor thanks to a dynein-dependent cytoplasmic mechanism, whereas FKBP51 increases the cytoplasmic pool of the receptor and decreases its nuclear pool. In this work it is demonstrated that the overexpression of the TPR domain of the immunophilin: (a) decreases the constitutive nuclear pool of the GR usually observed in the absence of hormone (Figs 2–4); (b) greatly delays the GR nuclear translocation upon cortisol addition (Figs 3 and 4); (c) favours the GR nuclear export (Fig. 5); (d) impairs the association of the receptor with NPC structures (Fig. 2); and (e) interferes with the GR anchorage to nuclear matrix-associated structures (Fig. 6). In other words, all the steps involved in the nuclear localization mechanism of the GR are affected by TPR factors, i.e. cytoplasmic transport, passage through the NPC, association to nuclear structures involved in the GR nuclear retention half-life process, and nuclear export mechanism. The model is summarized in Fig. 7.

These findings imply two main consequences: (1) the overall balance of TPR proteins capable of interacting with the receptor–Hsp90 heterocomplex plays a role in the final equilibrium responsible for the subcellular distribution of the receptor (and consequently, in its biological function), and (2) differential preferences to recruit certain TPR factors, a feature that is commanded by the ligand-binding domain of the receptor in a steroid-dependent manner (Gallo et al., 2007). The latter property may justify long-standing differences in receptor subcellular localization according to the cell type and the relative physiological condition. For example, the differential FKBP51:FKBP52 expression ratio of both immunophilins in different tissues may be related to the pleiotropic actions observed for GR.

The GR shows two major nuclear localization signals (Picard and Yamamoto, 1987), NLS1 and NLS2, the 28 amino acid bipartite NLS1 located in the hinge region of the receptor being the most efficient or potent to translocate the GR into the nucleus by interacting with importin-α/β1 heterodimers (Freedman and Yamamoto, 2004). On the other hand, NLS2 is more diffuse, comprises about 256 aa, and maps with the entire hormone binding domain of the GR (Picard and Yamamoto, 1987). While NLS1 mediates the rapid nuclear import of GR ($t_{0.5}$=4–6 min), NLS2-mediated nuclear import is much slower ($t_{0.5}$=45–60 min) and unable to produce full nuclear localization (Picard and Yamamoto, 1987). Note that the half-life for the translocation of the GR in TPR-overexpressing cells (Fig. 3B) is similar to that mediated by NLS2 only. This may be related to the fact that FKBP52 is able to interact with importin-β1 and the glycoprotein Nup62, an interaction that is prevented by the overexpression of the TPR peptide and should affect the NLS1-mediated nuclear translocation of the GR. Accordingly, we have previously reported that the knockdown of importin-β1 delays the nuclear import of the GR (Echeverria et al., 2009).

It is interesting to note that in the absence of cortisol, GFP-GR displays a partial level of colocalization with Nup62 and importin-β1 (Fig. 1); this is perhaps a consequence of the dynamic and permanent nuclear–cytoplasmic shuttling of the receptor. While the receptor accumulates in the nucleus after 20 min of cortisol treatment, it transiently increases the colocalization with Nup62 and simultaneously decreases that with importin-β1 in a TPR-competed fashion, suggesting a relevant role for FKBP52 during this process. This is in agreement with a previous publication where we demonstrated in a cell-free system that the reconstitution of the Nup62–Hsp90–FKBP52 complex from immunopurified Nup62 and reticulocyte lysate system enhances the interaction of the GR with the nuclear import machinery of the NPC (Echeverria et al.,

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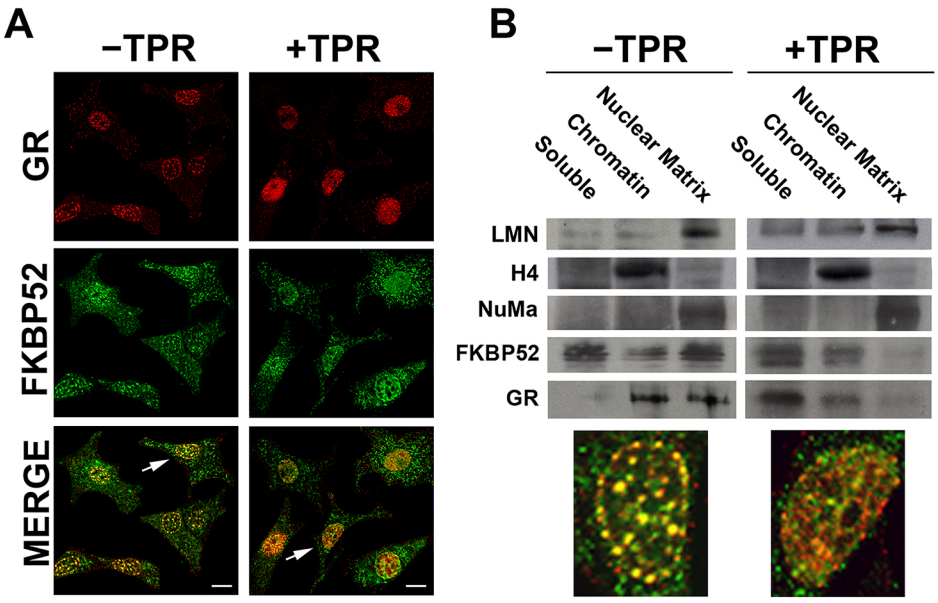

Fig. 6. FKBP52 is a nuclear matrix-associated protein. (A) The subcellular distribution of endogenous GR (red) and FKBP52 (green) was analysed by confocal microscopy in 3T3-L1 mouse fibroblasts transfected by electroporation with the TPR peptide of the immunophilin (+TPR) or empty vector (−TPR). Note in the TPR-overexpressing cells the lack of the nuclear punctuated pattern observed in control cells. The nuclei of those cells indicated by arrows are magnified at the bottom of panel B. (B) The nuclear matrix was purified following a standard biochemical fractionation method described in the literature (Fey et al., 1984). Lamin B (LMN) and nuclear matrix associated protein (NuMa) were used as nuclear matrix markers, and histone H4 was used as chromatin marker. Scale bars: 10 µm.

2009). In line with these assays, it is currently accepted that *in vitro* Nup assemblies serve as mimics of the selective permeability of the NPC (Zilman, 2018), leading to the concept that importins are also

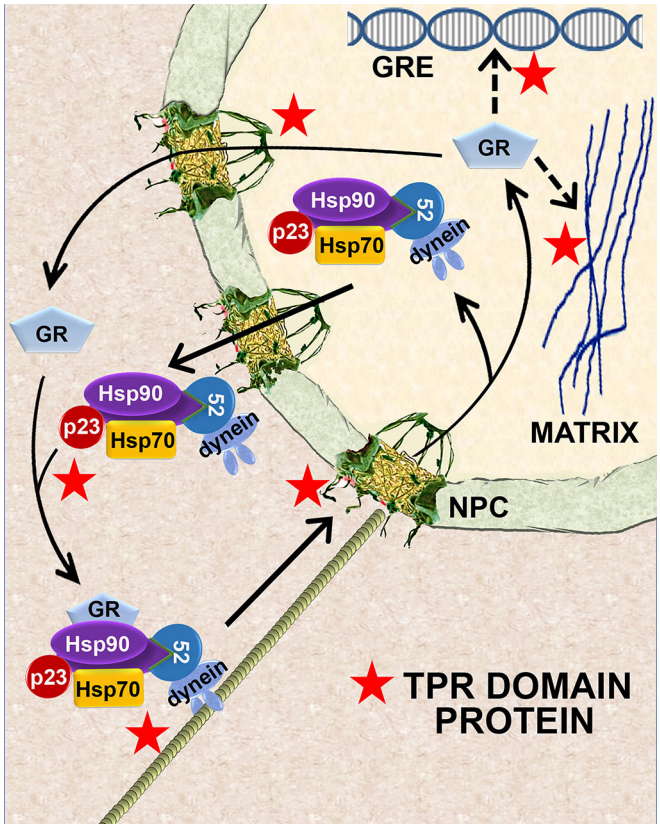

Fig. 7. TPR domain proteins regulate GR shuttling and the biological response. The integrated scheme shows the steps where TPR proteins (red stars) exert regulation on the GR mechanism of action, i.e. the cytoplasmic assembly of the GR–Hsp90 heterocomplex, the cytoplasmic transport of GR towards the nucleus, its interaction with nuclear pore complex (NPC) factors, transcriptional activity upon GRE (glucocorticoid responsive element) binding in target genes, GR association to nuclear matrix structures and nuclear export.

able to suppress the aggregation of cargoes and Nups (Jakel et al., 2002; Milles et al., 2013). Such activity involves shielding of basic patches on the cargo and requires a precise match between cargo and receptor. In view of the fact that an integral nucleoporin such as Nup62 is chaperoned by Hsp90–FKBP52, it could be entirely possible that the putative permeability barrier of the pore may also be regulated by these protein-protein interactions.

It is worthy to note that the association of TPR proteins with Nup62 should be dissociated with Hsp90 inhibitors since the linker is Hsp90. However, indirect immunofluorescence assays performed with intact cells treated with radicicol still show the presence of FKBP52 in the perinuclear ring stained with an anti-Nup62 antibody (Fig. S2). This suggests that the TPR immunophilin may also bind to the NPC structure in an Hsp90-independent manner, perhaps via other perinuclear structures. This explains why the competition experiments of Fig. 6 show that the overexpression of the TPR peptide abolishes the signal of FKBP52, indicating that most if not all types of association of the immunophilin with any structure of the nuclear envelope does involve its TPR domain.

Studies on the nuclear export mechanism of the GR showed contradictory results. While some studies have reported that inhibition of CRM1 by leptomycin-B enhances the nuclear localization of GR (Kakar et al., 2007; Savory et al., 1999), others have concluded that GR nuclear export is not dependent on CRM1, but due to an alternative mechanism (Black et al., 2001; Kumar et al., 2004; Liu and DeFranco, 2000). Our results show that leptomycin-B shows limited (if any) action on the GR nuclear export (Fig. 4) under conditions where it fully inhibited the export of RelA/p65, a known CRM1-dependent factor. We also demonstrated that the overexpression of the TPR peptide greatly accelerates GR nuclear export (Fig. 5A) indicating that a TPR protein should be involved in such mechanism. However, while leptomycin-B showed no effect by itself suggesting that CRM1 could not be involved in a significant manner, the TPR-dependent effect is prevented by the drug. This could be related to some other mechanism involving CRM1 associated with a TPR factor; in support of this, a proteomic study has proposed that CRM1 is chaperoned by Hsp90 (Falsone et al., 2005). To solve the conundrum, knockdown of CRM1 was performed (Fig. 5B), confirming that the regular export of GR is not affected by the lack of expression of the transporter. However,

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how leptomycin-B reverses the effect of TPR overexpression is still unsolved and two alternative mechanisms for nuclear export may exist, which in turn may be in line with the contradictory results described in the literature. A similar proposal was made for cells treated with UV radiation, where the JNK-mediated early export of GR appears to be mediated by the CRM1-dependent system and is consequently sensitive to leptomycin-B, but an additional late export mechanism seems to be insensitive to the drug and consequently mediated by other system(s) (Itoh et al., 2002). Calreticulin has also been postulated as potential export carrier of the GR by binding to a specific 11 aa sequence located in the DNA-binding domain between the two zinc fingers (Black et al., 2001).

It is accepted that leptomycin-B is targeted to the nuclear export signal-binding groove of CRM1 through covalent conjugation to a reactive cysteine residue (Sun et al., 2013). Nonetheless, the GR lacks a standard nuclear export sequence able to recognize that binding groove. Therefore, the mechanism of action for leptomycin-B on TPR may be different. It is also known that upon steroid withdrawal, Hsp90 plays a supporting role in the dissociation of the GR–DNA complex, also leading to faster GR export by facilitating GR passage through the NPC (Defranco, 2000; Liu and DeFranco, 1999; Pratt et al., 2004a). If this is the case, Hsp90-interacting factors carrying a TPR domain and sensitive to the effect of leptomycin-B should also be affected by overexpression of the TPR peptide and may justify our observations.

Another potential reason to explain the phenomenon is a non-specific effect of leptomycin-B. In fact, acute toxicity has been reported for concentrations even lower than 5 nM leptomycin-B after 1 h treatment (Mutka et al., 2009), and its clinical development was discontinued due to the significant toxicity observed in a phase I clinical trial without apparent medical efficacy (Newlands et al., 1996), all of which implies the putative existence of other still unknown side effects. Regardless of the exact mechanism of the CRM1 inhibitor, Fig. 6 suggests that nuclear GR may associate with the nucleoskeleton via FKBP52, a novel nuclear matrix-interacting protein that requires the TPR domain to achieve such anchorage. Consequently, TPR overexpression does affect the GR binding to nucleoskeleton structures favouring its nuclear export. We cannot rule out the possibility that nuclear speckles themselves are somehow disrupted by the overexpression of TPR peptides. The fact is that TPR proteins are indeed related to recruitment or anchorage of the GR to these structures. In line with this, other TPR domain proteins could also compete with these complexes affecting the GR subcellular redistribution and nuclear half-life. Indeed, this is a phenomenon previously reported when there is a variable FKBP51:FKBP52 expression rate (Banerjee et al., 2008; Galigniana et al., 2010b) and raises the possibility that the relative expression level of these immunophilins (and perhaps others) in different tissues may cause the pleiotropic actions observed for GR. Even more interestingly, the findings of the present study provide a link between the ability of the GR to regulate its subcellular localization and the potential of associated factors to affect this process, suggesting that the design of cell-permeable TPR-like peptides able to be specifically delivered to certain tissues or cell types could be an unconventional pharmacological tool for inhibiting Hsp90 client proteins such as the GR.

MATERIALS AND METHODS

Antibodies

The AC88 mouse monoclonal IgG against Hsp90 (cat. no. SPA-830; 1:500) and the N27F3-4 anti-72/73-kDa heat-shock protein monoclonal IgG (anti-Hsp70; cat. no. SPA-820; 1:2000) were from StressGen (Ann Arbor, MI). The rabbit polyclonal IgG (H114) anti-Hsp90α/β (cat. no. sc-7947; 1:1000), goat polyclonal IgG anti-lamin B (cat. no. sc-6216; 1:1000), mouse monoclonal IgG (H7) anti-CRM1 (cat. no. sc-137016; 1:2000) and goat polyclonal IgG anti-NuMA (cat. no. sc-18555; 1:250) were from Santa Cruz Biotechnologies (Santa Cruz, CA). Rabbit polyclonal IgG against FKBP51 (cat. no. PA1-020; 1:2000) and the BuGR2 mouse monoclonal IgG against the GR (cat. no. MA1-510; 1:1000) were from Affinity BioReagents (Golden, CO). The MG19 mouse monoclonal IgG against FKBP51 (1:50) was generated in the laboratory (Quintá et al., 2010). The UP30 rabbit antiserum against FKBP52 (1:1000) was a gift from William Pratt (University of Michigan, Ann Arbor, MI, USA). Mouse monoclonal IgG (clone Mab414) anti-Nup62 (cat. no. MMS-120R; 1:500) was from Covance (Berkeley, CA). Mouse monoclonal IgG anti-importinβ1 (cat. no. I-2534; 1:1000), mouse monoclonal M2 anti-Flag peptide IgG (cat. no. b-3111; 1:2000) and the secondary HRP-labelled counter-antibodies against L chain (cat. no. A2554 against mouse; A1949 against rabbit; both 1:4000) used for western blotting were from Sigma Chemical Co. (St Louis, MO). Fluorescent probe-tagged secondary antibodies for microscopy were from Jackson ImmunoResearch (West Grove, PA).

Cell culture and transfections

HEK293T (human embryonic kidney fibroblasts) and 3T3-L1 mouse fibroblasts were from ATCC (Manassas, VA). Cells were cultured using 10 cm in diameter plastic Petri dishes containing DMEM (Dulbecco′s modified Eagle′s medium from Gibco Argentina) supplemented with 10% (v/v) charcoal-stripped adult bovine serum (Internegocios, Argentina), and penicillin/streptomycin antibiotic cocktail (Sigma, St Louis, MO). For immunofluorescence studies, cells were plated on glass coverslips coated with poly-L-lysine. Cells were transfected using the polyethylenimine (PEI) transfection method when they reached about 30–40% confluence with 0.25 µg pEGFP-GR plasmid (a gift from Paul Housley, University of South Carolina School of Medicine, Columbia, SC, USA), and/or 0.25 µg pCMV6-Flag-TPR, or the pCMV6-Flag-TPR (R101A) mutant (a gift from Michel Chinkers, University of South Alabama, Mobile, AL, USA). In view of the fact that the TPR domain was used to compete with endogenous TPR domain proteins, it is important to emphasize that the structural properties of the peptide are identical to those of the native protein (Yang et al., 2005). The knockdown of CRM1 was achieved in HEK293T cells after transfection of a specific siRNA oligonucleotide (Santa Cruz Biotechnology, cat. no. sc-35116) following the manufacturer's instructions and has also been reported in the literature (Flegg et al., 2010; Gao et al., 2015). In all cases, the expression levels of all proteins of interest were always confirmed by western blot to ensure both the efficiency of the transfection procedure and the lack of influence of this on the endogenous expression of the other related proteins.

Cell fractionation

Biochemical fractionation of HEK293T cells into cytoplasmic and nuclear fractions was achieved as previously described (Gallo et al., 2011; Lagadari et al., 2016). For nuclear matrix purifications, 3T3-L1 fibroblasts were transfected by electroporation with 10 µg pCMV6-Flag-TPR or 10 µg empty pCMV6 vector. After 36 h, a standard fractionation protocol described in the literature was used (Fey et al., 1984) using lamin B and NuMA as nuclear matrix markers.

Immunoprecipitation

Cell extracts were washed with PBS and resuspended in BLH buffer (10 mM Hepes, pH 7.9, 1 mM EDTA, 1 mM EGTA, 20 mM Na_2MoO_4) supplemented with 0.1% NP40 and one tablet of Complete-Mini protease inhibitor mix/2 ml of buffer. After Dounce homogenization, one volume of CytoBuster™ protein extraction solution (Novagen, Darmstadt, Germany) supplemented with 1 µg/µl RNAsa and 1 µg/µl DNAsa was added to solubilize the GR bound to chromatin without disrupting protein complexes in those cases where homogenates were made from cells treated with steroid (Galigniana, 1998). Samples were incubated at 4°C for 1 h with rotation, and centrifuged at 4°C for 15 min at 10,000 ***g***. Supernatants (400 µl) containing ∼2.5 mg of protein were immunoprecipitated with 2 µg anti-GR mouse monoclonal antibody (clone BuGR2) or 2 µg anti-Hsp90 rabbit polyclonal H114 antibody and 15 µl Sepharose-conjugated protein A

(Sigma). After 3 h with rotation at 4°C, the pellets were washed five times with 1 ml TEGM buffer [TES at pH 7.6, 50 mM NaCl, 4 mM EDTA, 10% (v/v) glycerol] supplemented with 0.05% NP40. The immunocomplexes were boiled in SDS–β-mercaptoethanol-containing sample buffer, the proteins were resolved by SDS/PAGE, transferred to Immobilon-P membranes (Millipore, Burlington, MA), incubated with primary and then with the proper HRP-labelled secondary antibodies, and visualized by enhanced chemiluminescence (Salatino et al., 2006).

Immunofluorescence assays

Cells were fixed in 3.7% paraformaldehyde in PBS for 20 min, permeabilized with 0.5% Triton X-100 in PBS for 10 min and blocked for 1 h with 3% BSA in PBS. After incubation for 1 h at room temperature with primary antibodies followed by incubation for 1 h with the relevant fluorescent-conjugated secondary antibodies, the cells were mounted with Vectashield antifade medium (Vector Laboratories, Burlingame, CA) and visualized with a Zeiss LSM5 Pascal confocal microscope (Zeiss, Oberkochem, Germany) using a 100× oil objective with a NA of 1.35. Photographs were taken without exceeding the limits of the recording camera. To trigger GR nuclear translocation, 10 nM of cortisol (Steraloids Inc., Newport, RI) or 0.005% DMSO (vehicle) was added to the medium. For export experiments, cells were preincubated with 10 nM cortisol for 1 h, washed five times with DMEM, and cultured in DMEM/charcoal stripped serum for the periods of time indicated for each experiment. Inhibition of CRM1-dependent nuclear export was assayed by using leptomycin-B (LC Laboratories, Woburn, MA) at 10 ng/ml, and the drug was present in the medium during the entire period of the experiment. Cells were fixed in cold (−20°C) methanol, mounted as described above and visualized by confocal microscopy. Images were analysed with Image-Pro Plus Media Cybernetics software. The fluorescence intensities of the cytoplasm and the nucleus were quantified. Then the sum of both intensities was considered 100% of fluorescence, and the corresponding percentage for each compartment was calculated (five independent experiments with more than 100 cells counted for each time point). In some experiments, the subcellular localization of the GR was also visualized by western blot of cell extracts biochemically fractionated into nuclear and cytoplasmic fractions following a protocol described in previous studies (Gallo et al., 2011; Lagadari et al., 2016).

Live-cell imaging

HEK 293T cells were plated on poly-L-lysine coated coverslips in a 6-well plate and transfected with pEGFP-GR and pCMV6-Flag-TPR (or empty vector) at 1:10 plasmid ratio. The medium used for imaging was high glucose, Phenol Red-free DMEM medium containing 4 mM L-glutamine and 25 mM Hepes buffer (Invitrogen Corp, Carlsbad, CA), supplemented with 10% charcoal-stripped serum. GR nuclear translocation was triggered by the addition of 10 nM cortisol as described above.

Heterokaryon assays

For transient transfections involving interspecies heterokaryon formation, we employed the fusion method by polyethylene-glycol (PEG) shock described in the literature (Cazalla et al., 2005) with minor modifications. Briefly, HEK293T human cells grown on poly-L-lysine-coated coverslips in steroid-free medium were co-transfected with EGFP-GR and pCMV6 empty vector or pCMV6-Flag-TPR. Simultaneously, mouse L929 cells were also grown in a steroid-free medium and transfected with pCMV6 or pCMV6-Flag-TPR. The next day, HEK293T cells were exposed to 10 nM cortisol for 1 h and a 3-fold excess of L929 cells was seeded on the coverslips. After 3 h of co-incubation in a medium supplemented with 50 µg/ml cycloheximide (Sigma) and 10 nM cortisol, the culture medium was aspirated and 50% polyethylene-glycol (MW4000) in DMEM was added on the coverslips. After 90 s, the solution was rinsed off by five washes with DMEM, and cells were reincubated for 30 min in a steroid-free medium supplemented with 50 µg/ml cycloheximide and monitored by light microscopy. In our hands, fusions occur within a time frame of 10–15 min. Then, the medium was replaced with fresh steroid-free medium (plus cycloheximide) and incubation of the heterokaryons was continued for two additional hours prior to fixation. Cell nuclei were stained with DAPI to recognize the origin since murine nuclei are easily identified due to the presence of a high number of prominent chromatin speckles compared with human cell nuclei.

Statistical analysis

Data were expressed as the mean±s.e.m. Statistical significance was calculated by one-way ANOVA test followed by Duncan's multiple range validation. A *P*-value less than 0.05 was considered significant.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.D.G.; Methodology: G.I.M., P.C.E.; Software: G.I.M.; Validation: P.C.E.; Formal analysis: M.D.G.; Investigation: G.I.M., P.C.E.; Writing - original draft: M.D.G.; Supervision: M.D.G.; Project administration: M.D.G.; Funding acquisition: M.D.G.

Funding

The authors acknowledge financial support from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), The Universidad de Buenos Aires (UBACYT 20020170100558BA to M.D.G.), and the Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT) (PICT 2014-3433 and PICT 2016-0545 to M.D.G. and PICT 2016-2607 to G.I.M.).

Supplementary information

Supplementary information available online at
http://jcs.biologists.org/lookup/doi/10.1242/jcs.238873.supplemental

Peer review history

The peer review history is available online at https://jcs.biologists.org/lookup/doi/10.1242/jcs.238873.reviewer-comments.pdf

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