FRAP analysis of nucleocytoplasmic dynamics of the vitamin D receptor splice variant VDRB1: preferential targeting to nuclear speckles

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Although the key components of the cellular nuclear transport machinery have largely been characterized through extensive efforts in recent years, *in vivo* measurements of the kinetics of nuclear protein import/export are patently few. The present study applies the approach of FRAP (fluorescence recovery after photobleaching) to examine the nucleocytoplasmic flux of a novel human VDRB1 (vitamin D receptor B1) isoform in living cells. Through an N-terminal extension containing a consensus nuclear targeting sequence, VDRB1 is capable of localizing in nuclear speckles adjacent to SC-35 (35 kDa splicing component)-containing speckles as well as in the nucleoplasm, dependent on ligand. Investigation of VDRB1 nucleocytoplasmic transport using FRAP indicates for the first time that the VDRB1 has a serum-modulated, active nuclear import mechanism. There is no evi-

dence of an efficient, active export mechanism for VDRB1, probably as a result of nuclear retention. VDRB1 nuclear import in the absence of serum occurred more rapidly and to a greater extent to nuclear speckles compared with import to other nuclear sites. This preferential transport from the cytoplasm to and accumulation within nuclear speckles is consistent with the idea that the latter represent dynamic centres of VDRB1 interaction with other nuclear proteins. The results are consistent with the existence of specialized pathways to target proteins to nuclear subdomains.

Key words: fluorescence recovery after photobleaching (FRAP), nuclear export, nuclear hormone receptor, nuclear speckle, nucleus, vitamin D receptor B1 (VDRB1).

INTRODUCTION

The subcellular distribution of nuclear receptors such as steroid hormone receptor family members is a critical factor in their biological activity. Most are 'translocating receptors', moving from a principally cytoplasmic distribution in the absence of hormone to a predominantly nuclear localization in hormone-stimulated cells [1]. VDR (vitamin D receptor) translocates from the cytoplasm to the nucleus in hormone-, dose-, time-, temperature- and RXR (retinoid X receptor)-dependent fashion [2,3]. In contrast, receptors such as the oestrogen receptor and the A form of the progesterone receptor can be found in the nucleus even in the absence of hormone [4,5], whereas the thyroid hormone receptor has been shown to interact with specific DNA regulatory elements in the absence of a ligand, as well as repress selected genes in hormone-free cells [6–8].

In the absence of hormone, most steroid hormone receptors are associated with chaperones in a multimeric complex in the cytoplasm [9]. Ligand binding is believed to affect complex dissociation and enable the recognition of intrinsic NLSs (nuclear localization sequences) and nuclear export signals [10] by components of the nuclear import machinery [1], which determine the nuclear/cytoplasmic distribution.

We have previously characterized a 54 kDa VDR splice variant VDRB1, distinct from the previously recognized 48 kDa VDRA form, in human kidney as well as various osteoblastic, intestinal and kidney epithelial cell lines [11]. VDRB1 expression constructs show altered ligand-dependent transactivation compared with VDRA constructs when transiently transfected with a vitamin D responsive promoter [12]. Also, EGFP (enhanced

green fluorescent protein)-tagged VDRB1 exhibits localization in nuclear foci and partial co-localization with SC-35 (35 kDa splicing component)-containing speckles and a subset of PML (promyelocytic leukaemia) nuclear bodies in the absence of 1,25(OH)₂D₃ (1,25-dihydroxyvitamin D₃). In contrast, VDRA shows diffuse nuclear fluorescence, although, on 1,25(OH)₂D₃ treatment, both VDR isoforms exhibit diffuse nuclear localization [11]. The distinctive intranuclear compartmentalization and altered functional activity of VDRB1 are consistent with unique physiological roles, but no information is available with respect to the dynamics of VDRB1 transport between the different subcellular compartments and how this may relate to its function.

Many of the factors mediating transport between the nucleus and cytoplasm, including the transporters of the importin/exportin superfamily, have been identified over the last few years (see [13,14]). Physiologically relevant kinetic descriptions of the nuclear transport process, however, are scarce, with rate measurements largely restricted to semi-intact cell/diffusion-based *in vitro* systems that rely on high concentrations of nuclear-localizing substrates and transporters (e.g. [15–20]) or to computer simulations [20,21]. Several studies using the technique of FRAP (fluorescence recovery after photobleaching) have investigated the mobility of components of the nuclear lamina and chromatin within the nucleus [22,23], the splicing factor SF2/ASF [23,24], steroid hormone receptors [25,26], and even components of the nuclear pore complex/nuclear envelope [27,28]. However, very little kinetic analysis of the process of transport between the nuclear and cytoplasmic compartments has been performed in living cells.

Abbreviations used: CLSM, confocal laser scanning microscopy; DMEM, Dulbecco's modified Eagle's medium; EGFP, enhanced green fluorescent protein; FBS, fetal bovine serum; FRAP, fluorescence recovery after photobleaching; NLS, nuclear localization sequence; 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; PTHrP, parathyroid hormone-related protein; VDR, vitamin D receptor.

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The present study exploits recent technical advances [29–31] to examine the *in vivo* nucleocytoplasmic dynamics of VDRB1 for the first time, including long-term CLSM (confocal laser scanning microscopy) imaging and FRAP experiments on cells transfected to express transcriptionally active VDRB1–GFP fusion protein. We demonstrate that the rate and nature of nucleocytoplasmic flux of VDRB1 is altered by the presence of serum. Although an actively regulated nuclear import mechanism for VDRB1 is implicated, there is little evidence of rapid export from the nucleus. Importantly, the nuclear speckles in which VDRB1 localizes in the absence of hormone-containing serum appear to be ‘stable’ structural entities, owing to the fact that, subsequent to photobleaching, the fluorescence returns to the same position within the nucleus. Transport of VDRB1 from the cytoplasm to the nucleus in the absence of serum appears to be preferentially to these sites, consistent with the idea that the nuclear speckles represent specific sites of VDRB1 action. Importantly, the results parallel those of other recent studies [29,30] in implying the existence of specialized pathways to target proteins to nuclear subdomains.

MATERIALS AND METHODS

Tissue culture

COS-1 African green monkey kidney cells were maintained in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% (v/v) FBS (fetal bovine serum) containing approx. 80–300 pM 1,25(OH)₂D₃ (J. A. Eisman, unpublished work) was supplied by GIBCO Invitrogen (Carlsbad, CA, U.S.A.) at 37°C in 5% CO₂ atmosphere [29,30].

Transfection

COS-1 cells were passed through 22 mm diameter round coverslips for 2 days and then transfected with plasmids encoding either the VDRB1–GFP fusion protein [11] or EGFP as a control using Lipofectamine™ 2000 (GIBCO Invitrogen). The cells were transferred, 48 h after transfection, into an open perfusion microincubator cell chamber (PDMI-2, Medical Systems Corporation, Greenvale, NY, U.S.A.) and maintained at 37°C in Phenol Red-free DMEM buffered with 50 mM Hepes supplemented with 10% FBS.

CLSM imaging/FRAP experiments

For FRAP or imaging of cells in the absence of FBS, cells were incubated in medium lacking FBS for 1–4 h before experimentation. VDRB1–EGFP-expressing cells were identified using a CLSM (Bio-Rad Radiance 2000 confocal imaging system, Bio-Rad Microscience, Hemel Hempstead, U.K. and Nikon TE-300 microscope, Nikon Optical Company, Tokyo, Japan) equipped with 25 mW 488 and 512 nm argon lasers and 1.8 mW 543 nm green helium/neon laser, which allowed for a combined maximum illumination intensity of 0.3 mW at the point of focus within the sample. Cells were visualized by illumination with 30% total laser power at 488 nm. One image (five scans in Kalman mode) was collected before bleaching, and then an area covering at least 30% of the nucleus was selected by zooming 30-fold, or an area covering 30% of the cytoplasm was selected by zooming 8–10-fold. This area was then bleached by removing all barrier filters on the laser and allowing for maximum illumination of the area selected for 10 s. Images of cells were collected 20 s after bleaching and subsequent images acquired at 10 s intervals for 450 s, using detector and laser settings identical to those used before photobleaching. Monitoring the return of fluorescence 20 s after bleaching ensured that analysis was restricted to

Figure 1 Dynamics of VDRB1 in living cells

(A) Transiently transfected COS-1 cells expressing VDRB1–EGFP or EGFP in Phenol Red-free medium were visualized by CLSM in the absence or presence of serum (FBS, containing ligand, see the Materials and methods section) as indicated. (B) Quantitative data ($n=5$) for $F_{n/c}$ ($=F_n/F_c$, the ratio of the specific nucleoplasmic fluorescence F_n to the cytoplasmic fluorescence F_c) and $F_{sp/c}$ ($=F_{sp}/F_c$, the ratio of the specific fluorescence associated with the nuclear speckles F_{sp} compared with F_c) in the absence (1.5 h) or presence of serum for VDRB1–EGFP- or EGFP-expressing cells. Significant differences ($*P<0.05$) were observed between untreated (–FBS) and serum treated (+FBS) cells (Student’s *t* test). (C) Long-term imaging of VDRB1–EGFP-expressing cells as above in the absence of serum. Scale bar, 20 μ m. (D) Quantitative data from (C) showing the dynamics of VDRB1–EGFP nucleoplasmic and nuclear speckle (three separate structures) localization.

nucleocytoplasmic flux rather than intranuclear/intracytoplasmic lateral mobility (see also [29,30]). For long-term imaging, cells were maintained in serum-free, Phenol Red-free DMEM buffered with 50 mM Hepes, with CLSM scanning every 120 s for 1 h, using detector and laser settings identical with those used before the FRAP experiments. Quantification was performed using the ImageJ (NIH) and Kaleidagraph softwares [32–34].

RESULTS

VDRB1 localizes in nuclear speckles in the absence of serum

COS-1 cells were transfected with plasmids encoding either a VDRB1–EGFP fusion protein previously shown to be capable of transactivation in a ligand-dependent fashion [11] or EGFP as a control to examine the nucleocytoplasmic and intranuclear dynamics of VDRB1 using CLSM. Within 1 h of the removal of serum (containing ligand, see the Materials and methods section; all experiments ‘with serum’ correspond to the presence of ligand, and those without serum, the absence thereof), VDRB1 localized in nuclear speckles and displayed diffuse nuclear localization in its presence, as illustrated in Figure 1(A). Quantification by image analysis (Figure 1B) indicated that the nuclear/cytoplasmic ratio ($F_{n/c}$) for VDRB1–EGFP was approx. 3

(2.7 ± 0.1 , $n = 8$) in the presence of serum, significantly higher ($P < 0.05$) than in its absence ($F_{n/c} = 1.9 \pm 0.3$, $n = 10$). Surprisingly, fluorescence in the latter case was strongly associated with nuclear speckles, at levels about ten times (9.9 ± 3.0) those in the cytoplasm ($F_{sp/c} = F_{sp}/F_c$, the ratio of the specific fluorescence associated with the nuclear speckles F_{sp} to cytoplasmic fluorescence F_c) and more than four times higher than those in the nucleoplasm. This indicated that the ligand altered the localization of VDRB1 in the nucleus, increasing the movement from the cytoplasm to the nucleus, as well as from the nuclear speckles to the nucleoplasm. In comparison, the EGFP control indicated equal concentrations in the nuclear and cytoplasmic compartments ($F_{n/c} = 1$), with no evidence of nuclear speckle localization irrespective of the absence or presence of serum.

Long-term imaging using CLSM (Figure 1C) indicated that, although nucleoplasmic fluorescence did not change markedly over the 60 min time frame in the absence of serum (absence of ligand), the pattern of VDRB1–EGFP fluorescence in nuclear speckles fluctuated dynamically upwards and downwards as the protein moved in and out of the structures. Image analysis supported the idea of the dynamic nature of the VDRB1 nuclear speckles, as indicated by $F_{sp/c}$ values fluctuating between 12.5 and 2 for three different speckles; the slight trend towards a lower level of accumulation in all cases (Figure 1D) was due to a low level bleaching of the fluorescent signal with each CLSM imaging scan. Close examination revealed that, although the VDRB1 nuclear speckles displayed considerable dynamics during the course of 60 min, they remained essentially at the same position within the nuclear space. This observation, in conjunction with the FRAP experiments for the serum-starved cells (see below), suggests that the speckles are more than just random aggregations, but are structural entities, consistent with the results of others [24]. This supports the idea that VDRB1-containing nuclear speckles may represent sites of interaction with other nuclear proteins within a large macromolecular complex [11].

FRAP analysis of VDRB1 nuclear import; preferential transport to nuclear speckles in the absence of serum

By photobleaching regions within the nucleus in EGFP–VDRB1-expressing transfected cells, FRAP was applied to determine the rate and amount of nuclear import of VDRB1 from the cytoplasm in living cells for the first time in the absence (1–2 h) or presence of serum (Figure 2; movies representative of these experiments can be found at <http://www.med.monash.edu.au/biochem/research/projects/nuclearsig/realtime/VDRB1.html>). Our established experimental method [29,30] ensured that our analysis excluded rapid lateral movement within the nuclear compartment (see the Materials and methods section), and thus was restricted to the return of fluorescence due to transport from the cytoplasm to the nucleus. Figure 2(B) shows examples of the recovery curves, with the pooled data (Figure 2C, left panel) showing that the extent of recovery of nucleoplasmic fluorescence was significantly ($P < 0.05$) lower in the absence of serum (fractional recovery $47 \pm 3\%$, $n = 5$) than in its presence (fractional recovery $65 \pm 5\%$). The extent of recovery of fluorescence associated with the nuclear speckles in the absence of serum (fractional recovery $69 \pm 14\%$; Figure 2C, left panel) was comparable with that for nucleoplasmic fluorescence in the presence of serum. In all cases, the fractional recovery (mobile fraction) was 70% or less, indicating a significant fraction of immobile VDRB1, which increased to more than 50% for nucleoplasmic fluorescence in the absence of serum. This implied strongly that the serum (ligand) decreases VDRB1 binding to immobile nuclear components (see below). In contrast, analogous experiments on EGFP-expressing

cells indicated a complete return of nuclear fluorescence (see Figure 2C, left panel) at an approx. 2-fold slower rate ($t_{1/2} > 100$ s) than VDRB1 ($t_{1/2} < 60$ s) in either the absence or presence of serum (see Figure 2C, right panel), consistent with EGFP redistribution within the cell occurring by passive diffusion and in close agreement with previous estimations [29,30] (see also [35]) for experiments using a photoactivatable GFP derivative with essentially identical kinetics). It should be remembered that these $t_{1/2}$ values for the return of fluorescence represent an estimation of the transport rate from the cytoplasm to the nucleus, and not the lateral redistribution within the nucleus itself. The faster transport rates for VDRB1 compared with EGFP imply a facilitated nuclear import mechanism for VDRB1. The rates for the return of VDRB1 nucleoplasmic fluorescence were comparable in the absence or presence of serum ($t_{1/2} = 55 \pm 4$ and 59 ± 19 s respectively, $n = 5$), but were significantly lower ($P < 0.05$) than that for the return of fluorescence to nuclear speckles in the absence of serum ($t_{1/2} = 33 \pm 5$ s). Transport from the cytoplasm to and accumulation within nuclear speckles was thus faster and occurred to a greater extent compared with the rest of the nucleoplasm; hence, VDRB1 transport from the cytoplasm into the nucleus in the absence of serum occurred preferentially to nuclear speckles.

FRAP analysis of VDRB1 nuclear export

Although nuclear receptors are considered to be capable of continuous shuttling between nuclear and cytoplasmic compartments, the kinetics and mechanism of nuclear export *in vivo* is largely unknown [10]. Using photobleaching of regions of the cytoplasm of transfected VDRB1-expressing cells, FRAP analysis was performed to investigate the nuclear export of VDRB1 in the presence of serum. The return of cytoplasmic fluorescence through export from the nucleus took a significantly longer time ($t_{1/2} > 250$ s; Figure 3A) compared with that of EGFP-expressing control cells (146 ± 46 s), implying the lack or inhibition of an efficiently facilitated export mechanism, perhaps indicative of nuclear retention of VDRB1 by intranuclear interactions. The $t_{1/2}$ for EGFP cytoplasmic recovery was essentially comparable with that for nuclear recovery (Figure 2), validating the approach, and confirming the free diffusion between nuclear and cytoplasmic compartments for EGFP (see above; [29,30]). Measurement of nuclear fluorescence subsequent to cytoplasmic bleaching indicated that $83 \pm 1\%$ ($n = 5$) was retained, whereas only $33 \pm 1\%$ was retained for EGFP, again consistent with the idea of very slow facilitated nuclear export, and implying that nuclear VDRB1 quite probably spends a considerable amount of time bound to nuclear structures (Figure 3C).

DISCUSSION

Kinetic descriptions of nuclear protein import have largely been based on semi-intact cell systems, where ill-defined cytosolic extracts [15,16,18] are generally used. Even where transport has been successfully reconstituted using purified components in a few cases (e.g. [19,36]), non-physiologically high concentrations of nuclear import substrates are generally used. Furthermore, nuclear export studies are difficult using *in vitro* systems, owing to the fact that proteins initially need to be imported into the nucleus to provide a substrate for the export process. Most importantly, even though the microtubule system appears to play a key role in nuclear protein import *in vivo* as implied by several studies [30] (see [37–39]), the cytoskeleton is either lacking or severely damaged in these diffusion-based transport systems. Kinetic analysis of nuclear transport in living cells

Figure 2 FRAP analysis of the import of VDRB1-EGFP from the cytoplasm in transiently transfected untreated COS-1 cells and COS-1 cells treated with serum

(A) Transiently transfected COS-1 cells expressing VDRB1-EGFP or EGFP were bleached in the nucleus for 10 s (area indicated by the white box), and nuclear import was visualized at 10 s intervals over 400 s by CLSM to assess the return of fluorescence. Movie representations of these experiments [Fig2i.avi and Fig2ii.avi for VDRB1 in the presence or absence (1–2 h) of serum respectively; and Fig2iii.avi and Fig2iv.avi for EGFP in the presence or absence of serum respectively] are available at <http://www.med.monash.edu.au/biochem/research/projects/nuclearsig/realtimevideosVDRB1.html>. Scale bar, 20 μ m. (B) Quantitative results from (A) for fluorescence recovery, fitted according to the formulas $F_{n/c} = (F_{n/cmax})(1 - e^{-kt})$ or $F_{sp/c} = (F_{sp/cmax})(1 - e^{-kt})$, where k is the rate constant and t is time in seconds (30 s). The $t_{1/2}$ is indicated. (C) Pooled data ($n = 5$ cells) for the fractional recovery of nuclear fluorescence after photobleaching (left panel) and $t_{1/2}$ (right panel) were determined as in (B), where significant differences ($*P < 0.05$) were observed between the values for the return of nuclear VDRB1 for serum-treated cells (+) compared with untreated cells (–).

has been largely restricted to microinjection experiments using fluorescently labelled proteins (e.g. [16,17,34]), where the effect of damage and redistribution of cell components through the procedure is difficult to assess.

The present study represents an important contribution in that it constitutes the first insight into the dynamics of the nucleocytoplasmic as well as intranuclear dynamics of the novel VDR isoform VDRB1 in living cells; importantly, the results are for a GFP-VDRB1 fusion that is active for transactivation, dependent

on ligand, and where the fluorescence visualized/quantified in the FRAP experiments represents intact and not degraded protein, as shown previously [11]. Building on FRAP studies examining nuclear import/export of the signalling molecule PTHrP (parathyroid hormone-related protein) [29,30], our study demonstrates that VDRB1 is predominantly nuclear through a serum-modulated active import mechanism and the absence or inhibition of a rapid, active export mechanism. The nuclear speckles to which VDRB1 preferentially localizes in the absence of serum

Figure 3 FRAP analysis of the export of VDRB1–EGFP from the nucleus in transiently transfected COS-1 cells treated with serum

(A) Transiently transfected COS-1 cells expressing VDRB1–EGFP or EGFP were bleached in the cytoplasm for 20 s (area indicated by white box), and export from the nucleus visualized at 10 s intervals over 400 s by CLSM. Movie representations of these experiments (Fig3i.avi and Fig3ii.avi for VDRB1 and EGFP respectively) are available at <http://www.med.monash.edu.au/biochem/research/projects/nucleusig/realtime/movies/VDRB1.html>. Scale bar, 20 μ m. (B) Quantitative analysis from (A) for specific nuclear and cytoplasmic fluorescence fitted exponentially [29]. (C) Pooled data of the extent of nuclear retention (left panel) and fractional recovery of cytoplasmic fluorescence (right panel) after cytoplasmic bleaching, and determined as in (B), where significant differences (* $P < 0.05$) were observed between the values for VDRB1 and EGFP.

presumably represent sites of interaction with other nuclear proteins within a large macromolecular complex. In support of this concept, the immobile fraction of VDRB1 associated with nuclear speckles in the absence of serum (30%) differed from the immobile fraction associated with the nucleoplasm under the same conditions (50%). Furthermore, the return of mobile VDRB1 to the speckle sites after bleaching occurred more rapidly ($t_{1/2} = 33$ s) than its return to the nucleoplasm ($t_{1/2} \sim 60$ s). These observations are consistent with differential association of VDRB1 with distinct subnuclear structures in the absence of serum, demonstrated previously [11]. It will be interesting to determine whether these associations and their kinetics are regulated by post-translational modification such as phosphorylation. Of course, the FRAP technique applied here and previously [29,30] does not give information regarding the nature of the complexes within which VDRB1 appears to localize preferentially in the nucleus in the absence of a ligand. Our present hypothesis is that VDRB1 associates with other transcription factors and/or splicing factors, based on the fact that our previous study demonstrated co-localization to a significant extent of VDRB1 with PML nuclear bodies and SC-35 speckles in particular [11], but this would need to be confirmed by immunoprecipitation or other approaches.

Recent analysis (K. L. Sunn, S. J. Allison, L. Esteban, J. A. Eisman, D. A. Jans and E. M. Gardiner, unpublished work) indicates that VDRB1 nuclear import is probably mediated by the heterodimeric nuclear import receptor importin α/β , which

recognizes the N-terminal region that is absent from VDRA. Since VDRB1 transport from the cytoplasm to the nucleus appears to be preferentially to the nuclear speckles in the absence of serum as indicated, the potential role of importin α/β in mediating this, presumably in the absence of ligand, is of great interest. How this relates to the role of the previously characterized NLSs within VDRA [31,40,41], also present in VDRB1, in mediating ligand-independent nuclear import, and how this may regulate VDRB1 function, will be important to determine in the future.

That VDRB1 is targeted preferentially to nuclear speckles is of particular interest in that the PTHrP signalling molecule is analogously transported to a nuclear subcompartment, in this case the nucleolus [29,30]. The implication is that pathways specialized to target proteins to nuclear subdomains exist, the obvious advantages in terms of signalling being to ensure efficient intranuclear delivery to the sites of specific action. Since subnuclear domain targeting of VDRB1 appears to be mediated by importin α/β 1 (K. L. Sunn, S. J. Allison, L. Esteban, J. A. Eisman, D. A. Jans and E. M. Gardiner, unpublished work), whereas that of PTHrP is by importin β 1 independent of importin α [29,42,43], it seems clear that more than one importin-dependent nuclear import pathway can stimulate targeting of signalling molecules to subnuclear domains. The precise make-up and mechanistic details of the NLSs and pathways capable of mediating this targeting, presumably of critical importance to specialized nuclear function, will be the focus of future work in this laboratory.

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