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Intracellular calcium levels can regulate Importin-dependent nuclear import

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ABSTRACT

We previously showed that increased intracellular calcium can modulate Importin (Imp)β1-dependent nuclear import of SRY-related chromatin remodeling proteins. Here we extend this work to show for the first time that high intracellular calcium inhibits Impα/β1- or Impβ1-dependent nuclear protein import generally. The basis of this relates to the mislocalisation of the transport factors Impβ1 and Ran, which show significantly higher nuclear localization in contrast to various other factors, and RCC1, which shows altered subnuclear localisation. The results here establish for the first time that intracellular calcium modulates conventional nuclear import through direct effects on the nuclear transport machinery.

1. Introduction

Conventional nucleocytoplasmic transport in eukaryotic cells is regulated by members of the Importin (Imp) protein superfamily that mediate nuclear protein import and export through recognition of specific targeting signals [1]. Regulation of nuclear protein import, dependent on nuclear localisation signals (NLSs), is particularly important in cellular responses to extracellular signals modulating transcription and processes such as development [2,3]. Calcium (Ca^{2+}) signalling plays a critically important role in many cellular processes [4,5], with increasing evidence that Ca^{2+} plays a role in controlling subcellular localisation, and in particular nuclear-cytoplasmic translocation [6,7], although the precise mechanisms are not understood.

We recently showed that the sex determining factor SRY (Sex determining Region on the Y chromosome) possesses dual nuclear import mechanisms, dependent on two distinct NLSs, with Ca^{2+} acting as a switch between the two; increased intracellular Ca^{2+} enhanced calmodulin (CaM)-dependent nuclear import of SRY, and at the same time inhibited Impβ1-dependent nuclear import [6]. Similarly, Sweitzer and Hanover previously reported [7] a role for Ca^{2+} in modulating nuclear transport, with release of Ca^{2+} from the lumen of the nuclear envelope resulting in reduced nuclear import of SV40 T-ag (Simian Virus 40 T-antigen) via Impα/β. Several other studies point to a more general role of Ca^{2+} in regulating nuclear transport, with several groups reporting depletion of the Ca^{2+} from the cisternal space of the nuclear envelope in inhibiting passive diffusion as well as active transport into the nucleus [8–11]. However, recent studies have reported no effect of Ca^{2+} upon nuclear transport [12–15]. Likewise, although a link between the nuclear pore complex (NPC) conformation and Ca^{2+} has also been revealed [16], the reported effect of Ca^{2+} on NPC structural conformation are contradictory [16,17]. Clearly, although Ca^{2+} plays a role in regulating nuclear transport of proteins, the exact mechanisms by which its effects are exerted remain to be identified.

Here we set out to examine for the first time the possibility that intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) may modulate Imp-dependent nuclear transport generally. We find that elevated Ca^{2+} levels inhibit Impα/β1- and Impβ1-dependent nuclear protein import generally, and that the basis of this relates to direct effects on the subcellular localisation of specific components of the cellular nuclear transport machinery. The results have important implications for cell systems where Ca^{2+} signaling is a key player in determining response to cellular signals, differentiation/development, and a number of other processes.

2. Materials and methods

2.1. Cell culture

Cos-7 African green monkey kidney cell line was cultured in a 5% CO_2 humidified atmosphere at 37 °C, in Dulbecco's modified Eagle's medium (DMEM; ICN, Costa Mesa, CA, USA), supplemented

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with 10% heat inactivated fetal calf serum (FCS; CSL Ltd., Parkville, VIC, Australia), 1 mM L-glutamine, 1 mM penicillin/streptomycin and 20 mM Hepes.

2.2. Plasmid construction

Plasmids encoding the SV40- T-antigen (T-ag) 111–135 [18], TRF1 [19], SRY full length wild type [20] and GFP3 [21], as well as β-gal-PTHrP (82–108)-GFP and β-gal-PTHrP (94–108)-GFP [22] have all been previously described. GFP-aF10 (696–794) was generated by PCR as previously [20], using primers containing attB sites to enable derivation of the expression plasmid via recombination using Gateway™ Technology (Invitrogen, Carlsbad, CA, USA).

2.3. Transfection

Cos-7 cells were transfected 16 h post plating using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. Where indicated, cells were treated 16 h post transfection (pt) with 1 μM thapsigargin (Calbiochem, La Jolla, CA, USA) or 5 μM BAPTA-AM (Calbiochem) for 4 h prior, as previously [6], to CLSM imaging (see Section 2.5 below).

2.4. Immunofluorescence

Endogenously expressed proteins were visualised by CLSM (see below) in Cos-7 cells as previously, following fixation with

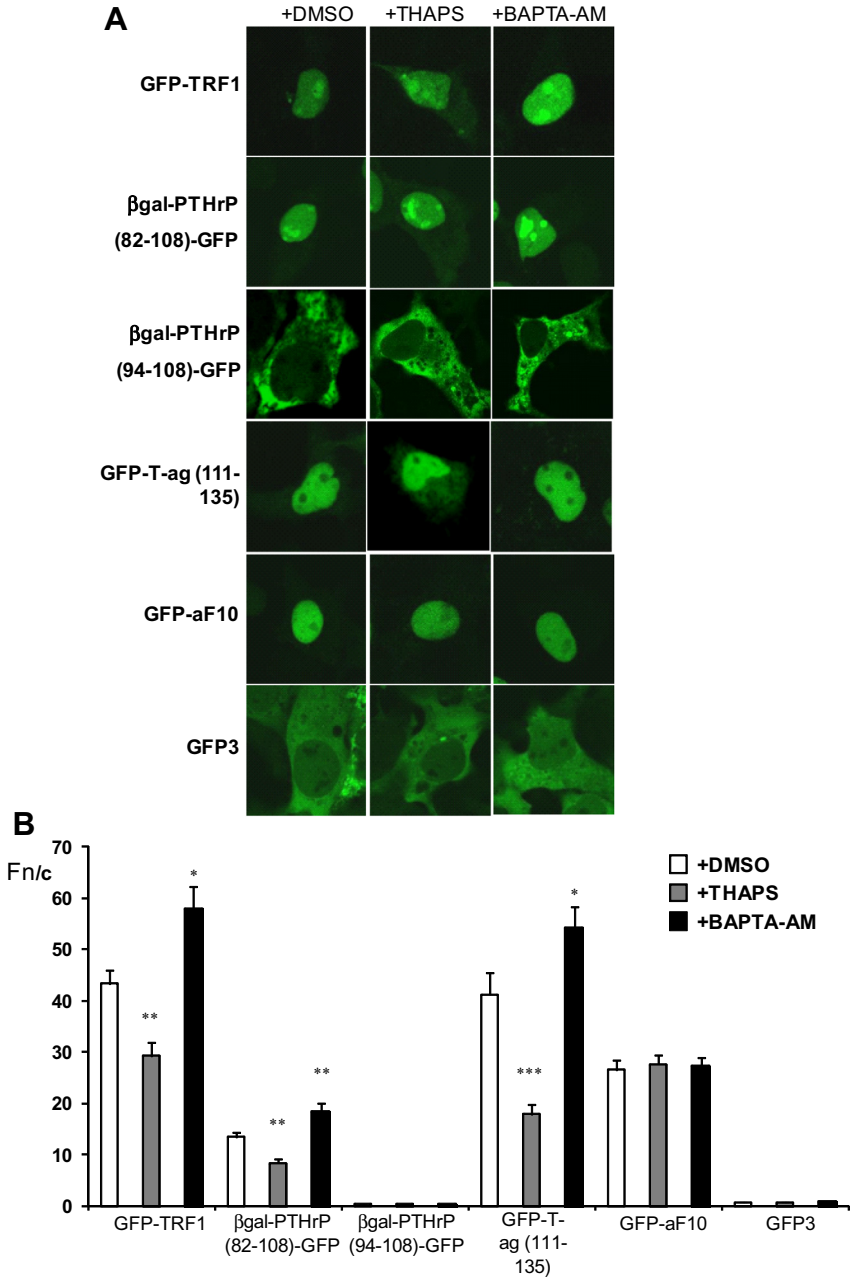

Fig. 1. Increased Ca^{2+} levels inhibit nuclear accumulation of ectopically expressed Impα/β1- and Impβ1-recognised nuclear import cargoes. Cos-7 cells were transiently transfected to express the indicated GFP-fusion proteins, treated without (DMSO) or with thapsigargin (THAPS) or BAPTA-AM as indicated 16 h post-transfection (p.t.), and then imaged live 4 h later by CLSM using a 40× water immersion objective. (A) Confocal images of typical cells indicating the subcellular localisation of the various GFP-fusion proteins. (B) Results for quantitative analysis, whereby CLSM images such as those in A were analysed using the Image J software for the extent of nuclear accumulation (the nuclear to cytoplasmic ratio, Fn/c; see Section 2). Results are for the mean ± SEM ($n > 23$), from a single typical experiment, from a series of > 3 similar experiments (see Table 1); p values from Student's t test (GraphPad Prism 5 software) are indicated; $^{***}p < 0.0001$, $^{**}p < 0.005$, $^{*}p < 0.01$ where values are significantly different in the presence of treatment with BAPTA-AM or THAPS compared to DMSO.

4% (w/v) paraformaldehyde. The fixed cells were incubated with mouse anti- Impβ1 (1:500, BD, Transduction Labs, Franklin Lakes, NJ, USA), Ran (1:100, BD), RCC1 (1:100, BD), mAb414 (NPC; 1:250, Abcam, Cambridge, MA, USA), Impα2 (1:200, BD), Impβ2 (1:250, BD), Exportin1 (1:250, BD), Rb (1:250, Cell Signalling Technology, Danvers, MA, USA) and, hnRNP A1 (1:750, Sigma, St. Louis, MO, USA), rabbit anti- SV40 T-ag (1:500, Santa Cruz, Santa Cruz, CA, USA), p53 (1:500, Cell Signalling Technology) and, Impβ3 (1:500, Santa Cruz), and, goat anti- Impα4 (1:250, Abcam) overnight at 4 °C, followed by either rabbit anti-goat, goat anti-rabbit or goat anti-mouse Alexa Fluor 488 (1:1000, Molecular Probes, Invitrogen) for 2 h at room temperature. Samples were mounted on coverslips in 4% propyl-gallate in PBS/glycerol (90% w/v) and imaged by CLSM (see below), where indicated, cells were treated with thapsigargin or BAPTA-AM (Calbiochem) for 4 h prior to fixation. FLUO 3/AM (Calbiochem) was used as a calcium indicator, as per the manufacturer's instructions.

2.5. CLSM/image analysis

Cells were imaged live by confocal laser scanning microscopy (CLSM; Bio Rad MRC-500, Richmond, CA, USA) using a 40× water immersion objective as indicated, with image analysis of digitized confocal images subsequently performed for populations of cells using the Image J (National Institutes of Health, Bethesda, MD, USA) public domain software. Nuclear (Fn) and cytoplasmic (Fc) fluorescence were quantified subsequent to the subtraction of autofluorescence, and used to derive the nuclear to cytoplasmic ratio (Fn/c), as previously [6,20,23].

3. Results

3.1. Altered Ca^{2+} levels modulate nucleocytoplasmic localization of ectopically expressed Impβ1- and Impα/β1-recognised nuclear import cargoes

We previously reported dual nuclear import mechanisms for the sex determining protein SRY, with increased $[Ca^{2+}]_i$ found to inhibit Impβ1-dependent nuclear transport [6]. To address the possibility that $[Ca^{2+}]_i$ may modulate nuclear protein import in general, we initially assessed an array of green fluorescent protein (GFP)-fused model nuclear localizing proteins, whose transport is conventionally dependent on either Impβ1 alone (telomere repeat binding factor 1, TRF1 [19] and parathyroid hormone-related protein, PTHrP [22]), or Impα/β (SV40 large tumor antigen, T-ag [3]), and compared their properties to those of SRY, as well as the chromatin remodeling factor aF10, which localizes in the nucleus through a pathway independent of Imps [24]. We assessed subcellular localization in living Cos-7 cells transfected to express the various GFP-fusion proteins (Fig. 1), following 4 h treatment, 16 h post-transfection (pt), with either thapsigargin (THAPS) or BAPTA-AM, agents which specifically increase or reduce $[Ca^{2+}]_i$ levels (see Supplementary Fig. 1), respectively [6]. CLSM analysis (Fig. 1A) revealed increased cytoplasmic fluorescence for the Impβ1-recognised nuclear import cargo GFP-TRF1 upon THAPS treatment, and increased nuclear fluorescence in response to BAPTA-AM. Similar results were observed for the much larger Impβ1-recognised β-gal-PTHrP(82–108)-GFP fusion protein.

Quantitative analysis of confocal files such as those in Figure 1A was performed to determine the nuclear to cytoplasmic ratio (Fn/c), results confirming those above; GFP-TRF1 showed significantly ($p = 0.0008$) almost 50% reduced and significantly ($p = 0.0399$) almost 40% increased nuclear accumulation upon THAPS or BAPTA-AM treatment, respectively (see Fig. 1B; Table 1). Similarly, β-gal-PTHrP(82–108)-GFP showed significantly ($p = 0.018$) 35% reduced nuclear accumulation in the presence of THAPS, as well as significantly ($p < 0.0001$) c. 40% increased nuclear accumulation in the presence of BAPTA-AM. A derivative of PTHrP [β-gal-PTHrP(94–108)-GFP] lacking an intact Impβ1-recognsied NLS [22] was also tested, with strong cytoplasmic localization observed (Fig. 1) that was not altered upon treatment with either THAPS or BAPTA-AM (Table 1), clearly implying that the effects of $[Ca^{2+}]_i$ are specific to signal-dependent nuclear transport.

Analysis for the NLS-containing Impα/β-recognised GFP-T-ag(111–135) protein also showed significantly ($p < 0.0001$) reduced (c. 40%) nuclear accumulation in the presence of THAPS treatment, as well as a significant ($p = 0.0399$) increase (13%) in nuclear localisation in the presence of BAPTA-AM treatment. The control protein SRY showed significantly ($p < 0.0001$) reduced nuclear accumulation in the presence of THAPS (c. 40%) and BAPTA-AM (c. 35%), as previously [6] (see Table 1). Importantly, no change in nuclear accumulation in either the absence or presence of THAPS or BAPTA-AM were observed for GFP-aF10 (Fig. 1), implying the specificity of the results for Imp-dependent transport, with completely analogous results observed for the c. 80 kDa tandem triple GFP molecule, GFP3, which lacks an NLS sequence; no changes to GFP3's cytoplasmic localization were observed upon increasing or decreasing $[Ca^{2+}]_i$ levels, implying no alteration to the passive diffusion properties of the nuclear pore. Thus, the effect of Ca^{2+} on nuclear import is specific to Imp-dependent/NLS-dependent nuclear import, and does not relate to direct effects on the properties of the nuclear pore.

Table 1
Summary of pooled data for the extent of nuclear accumulation of ectopically expressed GFP-fusion proteins in the absence or presence of BAPTA-AM or THAPS.

Protein (*n*)	Treatment	% Fn/c[a]	*p* value[b]
SRY (4)	+DMSO	100	
	+BAPTA-AM	65 ± 3	<0.0001
	+THAPS	60 ± 3	<0.0001
TRF1 (3)	+DMSO	100	
	+BAPTA-AM	143 ± 16	0.0399
	+THAPS	53 ± 8	0.0008
βgal-PTHrP (82–108) (3)	+DMSO	100	
	+BAPTA-AM	138 ± 2	<0.0001
	+THAPS	65 ± 9	0.018
βgal-PTHrP (94–108) (3)	+DMSO	100	
	+BAPTA-AM	101 ± 2	–
	+THAPS	98 ± 2	–
T-ag (111–135) (6)	+DMSO	100	
	+BAPTA-AM	113 ± 2	0.0002
	+THAPS	59 ± 6	<0.0001
aF10 (696–794) (3)	+DMSO	100	
	+BAPTA-AM	98 ± 4	–
	+THAPS	100 ± 2	–
GFP3 (4)	+DMSO	100	
	+BAPTA-AM	104 ± 2	–
	+THAPS	102 ± 4	–

[a] Data collated from transfection assays such as those shown in Fig. 1, representing the mean ± SEM (*n* in parentheses) of the percentage of the value relative to accumulation in the presence of DMSO, %Fn/c.
[b] *p* values (Student *t*-test, performed using the GraphPad Prism 5 software) are indicated where there were significant differences between values compared to DMSO.

3.2. Altered Ca^{2+} levels modulate nucleocytoplasmic localization of endogenous nuclear import cargoes

To confirm the relevance of the above results to endogenous nuclear import cargoes not under conditions of ectopic expression, the subcellular localisation of several endogenously expressed pro-

teins – the tumor suppressors p53 and pRb (retinoblastoma susceptibility factor), the viral protein SV40 T-ag, constitutively expressed in Cos-7 cells, as well as the nuclear ribonucleoprotein A1 (hnRNP A1) – were examined after treatment with THAPS or BAPTA-AM (Fig. 2). Endogenously expressed T-ag showed changes in localization comparable to those observed above for ectopic expression (Fig. 1), with significantly ($p = 0.003$) 23% reduced nuclear accumulation in the presence of THAPS, and 24% increase in nuclear accumulation in the presence of BAPTA-AM ($p = 0.0018$). Similarly, the Impα/β-recognised pRb [25] and p53 [26], showed significantly ($p < 0.0022$) 30% and 40% reduced nuclear accumulation in the presence of THAPS, respectively, although p53 uniquely also showed a significant ($p = 0.0074$) 43% reduction in the presence of BAPTA-AM (Fig. 2), which may reflect upon the ability of Ca^{2+} to regulate p53 nuclear import via S100B [27]. Interestingly, the Impβ2-dependent hnRNP A1 [3,28] showed no changes in subcellular localisation in the presence or absence of THAPS or BAPTA-AM (see Fig. 2).

Together with the results for ectopically expressed cargoes (Fig. 1), these results imply that increased $[Ca^{2+}]_i$ levels (THAPS treatment) specifically inhibit Impα/β1- as well as Impβ1-dependent nuclear protein import, with decreased Ca^{2+} levels (BAPTA-AM treatment) able to elevate nuclear accumulation of a number of Impα/β1- and Impβ1-recognised cargoes, with no effects observed for the Impβ2-dependent cargo in the presence of THAPS or BAPTA-AM. Ca^{2+} levels can thus modulate Imp-dependent nuclear import, implying effects on the nuclear import mechanism itself.

3.3. Ca^{2+} levels can modulate the localisation of key components of the cellular nuclear transport machinery

As a first step to determine the basis of the effects of altered Ca^{2+} levels on nuclear transport, we assessed the subcellular localization, by immunofluorescence, of a number of key components of the nuclear transport machinery in the absence and presence of treatment with THAPS or BAPTA-AM (Fig. 3). Increased nuclear localisation of Impβ1 and Ran were observed (see Fig. 3A), with quantitative analysis indicating significant ($p < 0.0222$) increases in nuclear accumulation (27% and 22%, respectively) in the presence of THAPS. Although only a slight effect of BAPTA-AM treatment was observed for Impβ1, increased cytoplasmic staining as well as a significant ($p = 0.0389$) c. 15% reduction in nuclear accumulation was observed for Ran. Intriguingly, the Ran guanine nucleotide exchange factor RCC1 also showed increased cytoplasmic localisation, with significantly ($p = 0.0077$) reduced (c. 22%) nuclear accumulation in the

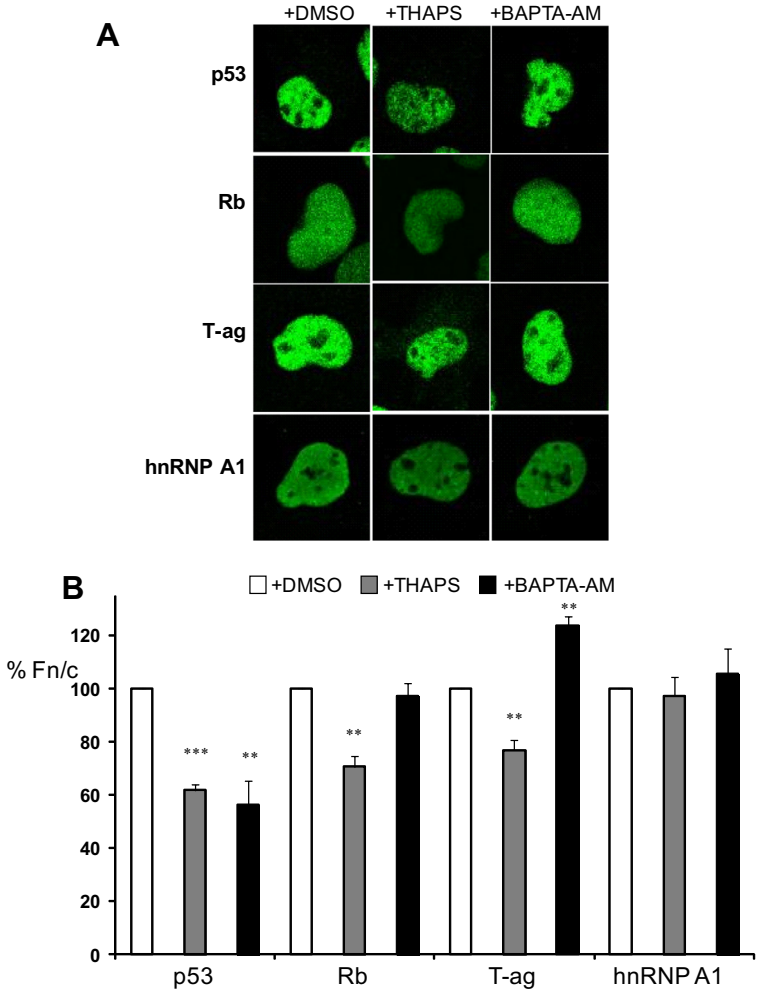

Fig. 2. Increased Ca^{2+} levels inhibit nuclear accumulation of endogenously expressed Impα/β1- and Impβ1-recognised nuclear import cargoes. Cos-7 cells were treated without (DMSO) or with THAPS or BAPTA-AM for 4 h, fixed and then processed for immunofluorescence using specific antibodies as indicated (see Section 2). (A) Confocal images of typical cells indicating the localisation patterns of the various endogenously expressed proteins. (B) Results for quantitative analysis of images ($n > 23$) such as those in A, as per legend to Fig. 1B. Results are for the mean ± SEM ($n = 3$) of the percentage Fn/c relative to accumulation in the presence of DMSO for the various proteins in the presence of THAPS or BAPTA-AM; p values are indicated as per legend to Figure 1B.

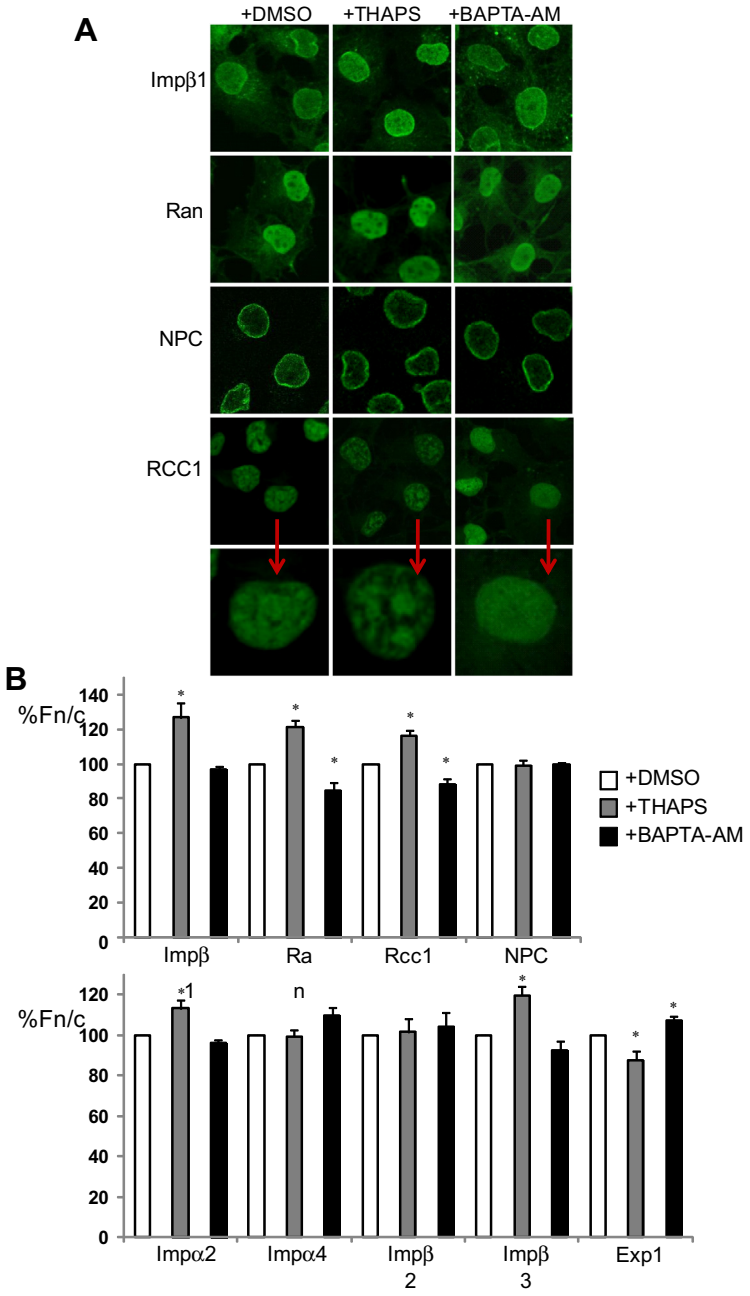

Fig. 3. Varying Ca^{2+} levels modulate subcellular localisation of specific components of the cellular nucleocytoplasmic machinery. Cos-7 cells were treated without (DMSO) or with THAPS or BAPTA-AM for 4 h and then fixed and processed for immunofluorescence using specific antibodies as indicated (see Section 2). (A) Confocal images of typical cells indicating the localisation patterns of the various proteins. Red arrows denote cells that are featured in detail panels highlighting subnuclear localisation of RCC1. (B) Results for quantitative analysis of images ($n > 24$) such as those in A, as per legend to Fig. 1B. Results are for the mean ± SEM ($n = 3$) of the percentage Fn/c relative to accumulation in the presence of DMSO for the various proteins in the presence of THAPS or BAPTA-AM; values significantly different in the presence of treatment with BAPTA-AM or THAPS compared to DMSO are indicated. $^{***}p < 0.0001$, $^{**}p < 0.005$, $^{*}p < 0.05$.

presence of BAPTA-AM and significantly ($p = 0.0364$) increased nuclear localisation (c. 17%) quantified in the presence of THAPS. Furthermore, whilst treatment with BAPTA-AM resulted in diffuse localisation of RCC1 in both nuclear and cytoplasmic compartments, changes in subnuclear localisation were observed upon treatment with THAPS, with larger aggregates formed compared to in the absence of treatment (see Fig. 3A). These results provide evidence that changes in $[Ca^{2+}]_i$ can lead to the mislocalisation of key factors of Imp-dependent nuclear transport, which result in changes to nuclear localisation of Impα/β- and Impβ-dependent cargoes as observed above (Fig. 1 and Fig. 2).

Other nuclear import proteins such as Impα2 and Impβ3, in similar fashion to Impβ1, showed significantly ($p < 0.0307$) increased nuclear accumulation in the presence of THAPS (13% and 20%, respectively), with no change in the presence of BAPTA-AM, whilst the nuclear export protein Exportin1 (CRM1), showed significantly ($p = 0.0455$) 22% reduced nuclear accumulation in the presence of THAPS and increased nuclear accumulation in the presence of BAPTA-AM (c. 7%, $p = 0.0336$). Intriguingly, Impα4 showed no change in subcellular localisation in the presence of either THAPS or BAPTA-AM. Similarly, Impβ2 also showed no change in its subcellular localisation upon treatment with THAPS

or BAPTA-AM, which may explain the lack of change in subcellular localisation quantified for hnRNP A1 above (see Fig. 2). No change in subcellular localisation was also observed for nuclear pore complex (NPC) proteins upon treatment with THAPS and BAPTA-AM (Fig. 3), indicating once again that the effects observed for changes in nuclear accumulation for cargo proteins do not appear to be the result of alterations in the nuclear pore.

The results here imply for the first time that either increasing or reducing $[Ca^{2+}]_i$ levels can have a significant impact on Imp-dependent nuclear transport, presumably in part, as a direct result of effects on the subcellular localisation of key components of the nuclear transport machinery, and in particular Impβ1, Ran and RCC1. The increased nuclear localisation of Impβ1 observed in the presence of elevated $[Ca^{2+}]_i$, appears to result in reduced levels of Impβ1 in the cytoplasm available for cycles of nuclear protein import, presumably representing the basis of the reduced nuclear import of proteins such as T-ag, PTHrP and TRF1 in the presence of THAPS (Fig. 1 and Fig. 2). Altered localisation of Impβ1 may in turn have resulted from reduced nuclear localisation of RCC1, which almost certainly impacts on the efficiency of Ran in driving nuclear transport generally [3]; Ran also showed reduced nuclear accumulation in the presence of elevated Ca^{2+}, as well as higher levels of protein in the nucleus upon reduced $[Ca^{2+}]_i$, which may explain the increase in nuclear accumulation observed for TRF1, PTHrP and T-ag in the presence of BAPTA-AM.

4. Discussion

The results here show for the first time that changes in intracellular Ca^{2+} can significantly impact on Imp-dependent nuclear transport, with elevated Ca^{2+} levels inhibiting Impα/β1- and Impβ1-dependent nuclear protein import generally. The basis of this appears to be through direct effects on the subcellular localisation of specific components of the cellular nuclear transport machinery. The implications of this study are widespread, especially in terms of how the cellular nuclear transport machinery may function in cells from muscle to neurons where Ca^{2+} flux is central to cell response [4]. That calmodulin can substitute for Imp-dependent nuclear import for a number of cellular proteins critical during development (e.g. the SOX family of chromatin remodeling factors) [6,20,29,30] indicates that other pathways/mechanisms can compensate for reduced Imp-dependent activities, but exactly how this may play out remains to be determined. This intriguing question is the focus of future work in this laboratory.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.06.047.

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