

The JNK/AP-1 pathway upregulates expression of the recycling endosome *rab11a* gene in B cells transformed by *Theileria*

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Summary

Lymphocyte transformation induced by *Theileria* parasites involves constitutive activation of c-Jun N-terminal kinase (JNK) and the AP-1 transcription factor. We found that JNK/AP-1 activation is associated with elevated levels of Rab11 protein in *Theileria*-transformed B cells. We show that AP-1 regulates *rab11a* promoter activity in B cells and that the induction of c-Jun activity in mouse fibroblasts also leads to increased transcription of the endogenous *rab11a* gene, consistent with it being an AP-1 target. Pharmacological inhibition of the JNK pathway reduced Rab11 protein levels and endosome recycling of transferrin receptor (TfR) and siRNA knockdown of JNK1 and Rab11A levels also reduced TfR surface expression. We propose a model, where activation of

the JNK/AP-1 pathway during cell transformation might assure that the regulation of recycling endosomes is co-ordinated with cell-cycle progression. This might be achieved via the simultaneous upregulation of the cell cycle machinery (e.g. cyclin D1) and the recycling endosome regulators (e.g. Rab11A).

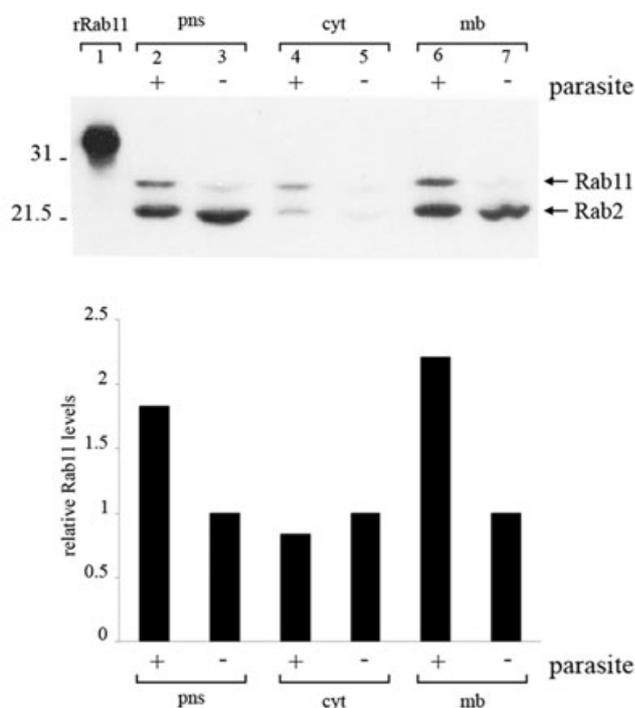
Introduction

Rab proteins form a subgroup of the large family of Ras-related GTPases that are essential components of the vesicular trafficking machinery (Seabra *et al.*, 2002). Rab proteins exist in a membrane-associated, GTP-bound form, which can be reversibly released to give a cytosolic GDP-bound form associated with a chaperone protein, RabGDI (Rab-specific GDP dissociation inhibitor) (Pfeffer, 2001). Endocytosis, an essential process common to all eukaryotic cells, permits uptake of macromolecules, modulation of cell-surface receptor expression, and general maintenance of homeostasis (Maxfield and McGraw, 2004). Several proteins have been described as regulators of mammalian endocytosis and it is clear that Rab5 and Rab11 in particular play essential roles (Maxfield and McGraw, 2004). Rab5 is located in early endosomes and controls the early steps of endocytosis and homotypic fusion of these organelles (Woodman, 2000). The role of Rab11 in regulating recycling endosomes is less well understood. However, Rab11A is clearly involved in the late recycling of transferrin receptor (TfR) back to the plasma membrane (Ullrich *et al.*, 1996; Ren *et al.*, 1998; Trischler *et al.*, 1999), via interaction with its coupling protein FIP2 (Lindsay and McCaffrey, 2002). Rab11A is also involved in transport events between recycling endosomes and the Trans-Golgi-Network (Wilcke *et al.*, 2000). In contrast, Rab11B does not appear to be involved in TfR recycling (Lapierre *et al.*, 2003).

Theileria parasites infect cattle and, in the absence of appropriate treatment, induce a rapidly lethal leukaemia-like disease (reviewed in Dobbelaere and Rottenberg, 2003). *Theileria*-infected lymphoblasts behave as transformed cell lines and when injected subcutaneously into irradiated athymic mice develop into metastatic tumours (Fell *et al.*, 1990). We have recently shown that phosphatidylinositol 3-kinase (PI3-K) (Baumgartner *et al.*,

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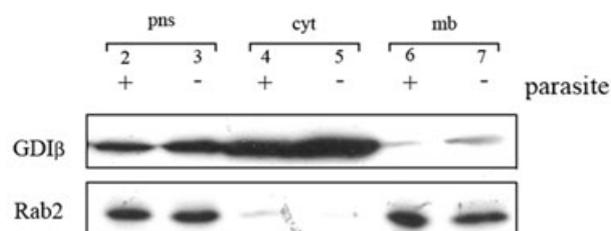


Fig. 1. Western blot analysis of the expression and subcellular distribution of Rab11 in *T. annulata*-infected B cells. The levels of Rab11 were compared with Rab2 taken as a loading control. We purified post-nuclear supernatants (pns, obtained after mechanical breaking of cells), cytosolic fractions (cyt), or membrane fractions (mb) from non-infected (BL3) and infected B cells (TBL3, shown +).

A. Top: A comparison in the distribution of Rab11 and Rab2 revealed increased amounts of Rab11 (compare lanes 2 and 3) and elevated Rab11 associated with the membrane fraction (compare lanes 6 and 7) in infected TBL3 cells. The slightly slower mobility of the control rRab11 recombinant proteins (A, lane 1) is due to the histidine-tag. Bottom: The amount of Rab11 was estimated by densitometry, normalized with regard to Rab2 amounts in BL3 cells for each fraction and presented as relative Rab11 levels.

B. The efficiency of subcellular fractionation was estimated by monitoring the predominantly soluble rabGDI compared with largely membrane-associated Rab2. Lanes, 2, 4 and 6 represent extracts from infected TBL3 cells (parasite +) and lanes, 3, 5 and 7 were from non-infected (parasite -) BL3 cells.

2000) and Src kinases (Baumgartner *et al.*, 2003) contribute to the permanent induction of the transcription factor AP-1 that characterizes transformation of *Theileria*-infected lymphocytes (Galley *et al.*, 1997; Chaussepied *et al.*, 1998). AP-1 induction appears to pass exclusively via the JNK kinase pathway and inhibition of JNK leads to apoptosis of infected B cells, where as interfering with AP-1 transcriptional activity reduces tumour invasiveness (Lizundia *et al.*, 2005; 2006).

AP-1 is a dimer of Fos and Jun proteins implicated in the regulation of cell proliferation, transformation and apoptosis (Jochum *et al.*, 2001). AP-1 target genes involved in both cell cycle progression (e.g. cyclin D1), and tumour cell invasion (e.g. collagenase/MMP-1) have been identified. However, we are far from understanding the entire AP-1-regulated transcriptional programme that is important in cell transformation and cancer progression. It has been shown that *Theileria*-infected T lymphocytes have increased amounts of TfR (CD71) on their surface

(Naessens *et al.*, 1996). Given the established role of Rab11A in TfR recycling in *Theileria*-infected B cells (Baumgartner *et al.*, 2005), we examined whether *rab11a* expression was regulated by AP-1 upon *Theileria*-induced transformation.

Results

Upregulation of Rab11 expression and rab11a promoter activity in Theileria-transformed B cells

We examined the expression of Rab proteins in *Theileria annulata*-infected B cells, in which transformation has been linked to upregulation of AP-1, and compared Rab11 levels with those observed for Rab2 in the isogenic non-infected B cell line BL3. We found that Rab11 protein levels were clearly elevated in infected lymphocytes compared with isogenic non-infected B cells (Fig. 1, lanes 2, 4 and 6). This appears to be specific for Rab11, as the

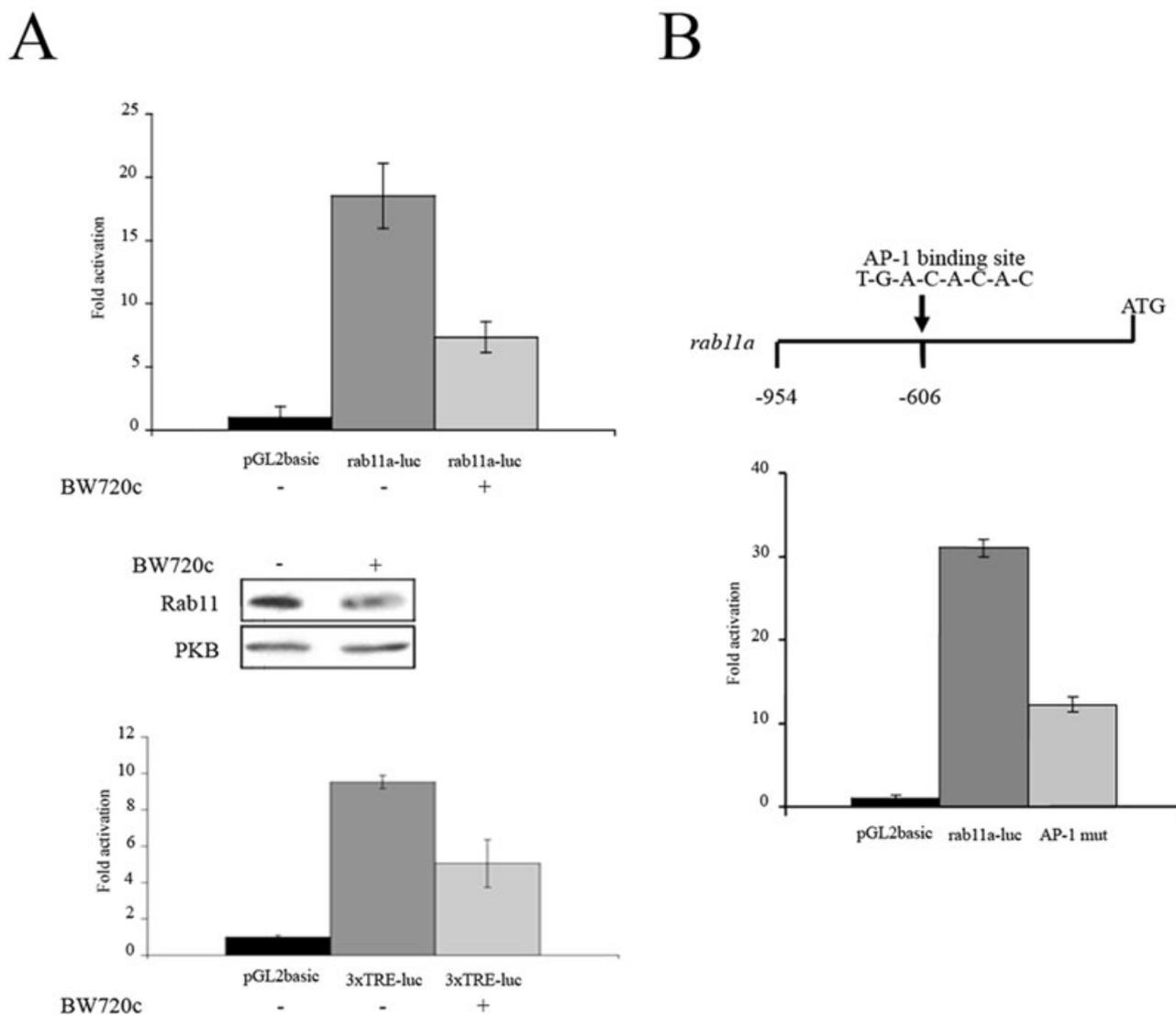


Fig. 2. *Theileria*-induced AP-1 regulates *rab11a* promoter activity. A. Top: The *rab11a* promoter is constitutively active in *T. parva*-transformed B cells and BW720c treatment leads to a threefold reduction. Middle: BW720c-induced parasite death also leads to a reduction in Rab11 protein levels compared with PKB in *T. parva*-infected B cells. Bottom: Constitutive AP-1 activity is parasite-dependent, as 3XTRE-driven luciferase is higher than pGL2basic and BW720c-induced parasite death reduces 3XTRE-driven activity by almost threefold. B. Top: A schematic of the 954 bp fragment of the *rab11a* promoter with the position and sequence of the potential AP-1 binding site. Bottom: The luciferase activity obtained with 'wild-type' *rab11a* and *rab11a* with the mutated AP-1 site (AP-1-mut) are compared with the minimal pGL2basic promoter activity 24 h post transfection.

levels of Rab2 (that serve as a loading control) and Rab6 (Baumgartner *et al.*, 2005) were essentially unaltered between infected TBL3 and non-infected BL3 cells. We observed that not only was there more total Rab11, but also that the majority of Rab11 was associated with the membrane fraction (see densitometry scan analysis of Rab11 levels associated with membrane, Mb) indicating that it is in the GTP-bound active form. The fractionation of membrane from cytosol was verified by the distribution of rabGDI β compared with Rab2 (Fig. 1B).

To test whether the high Rab11 protein levels in transformed B lymphocytes correlate with enhanced activity of the *rab11a* promoter, we cloned a 1 kb fragment of the putative human *rab11a* promoter upstream of the luciferase reporter gene (see *Experimental procedures*). We first established that the *rab11a* promoter was constitutively active in *Theileria*-infected B cells (Fig. 2A, top). This activity was largely parasite-dependent, as BW720c treatment led to a threefold decrease. This mimics the reduction in AP-1 activity upon drug-induced parasite

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death (Fig. 2A, bottom). BW720c treatment results in a similar decrease in Rab11 protein levels (Fig. 2A, middle panel).

Thus, the *rab11a* promoter activity parallels the parasite-induced constitutive activation of AP-1 (Chaussepied *et al.*, 1998). We therefore examined the proximal promoter region upstream of the *rab11a* gene in the human genome and a computer-assisted search for transcription factor binding sites revealed the presence of a potential AP-1 binding site (5'-TGACACA) 606 base pairs (bp) upstream of the *rab11a* open reading frame (Fig. 2B). To test directly the contribution of the AP-1 binding site in the *rab11a* promoter, we specifically mutated the site and compared promoter activity with the 'wild-type' *rab11a* promoter (Fig. 2B). Specific mutation of the AP-1 site reduces threefold the *rab11a*-driven luciferase activity compared with the 'wild-type' promoter. Thus, mutating the AP-1 site resulted in a level of reduction similar to that observed upon drug-induced parasite death (Fig. 2A). These results suggest that *Theileria* transformation leads to upregulation of the *rab11a* promoter activity and that this is mostly due to induction of AP-1 and its binding to the TRE site in the *rab11a* promoter.

c-Jun induction increases endogenous *rab11a* transcription in fibroblasts

To obtain further confirmation that *rab11a* expression is regulated by the AP-1 transcription factor, we measured the amount of endogenous murine *rab11a* transcripts in fibroblast cells expressing an inducible c-Jun–oestrogen receptor (CER) fusion protein (Bossy-Wetzel *et al.*, 1997). NIH3T3-CER cells were grown overnight in low-serum conditions and c-Jun activity was then induced with oestrogen, as previously described (Bossy-Wetzel *et al.*, 1997). The induction of *rab11a* mRNA was compared with *cyclinD1*, an established AP-1 target gene (Bakiri *et al.*, 2000) and 18s mRNA levels were used as a control for standardization. Upon CER induction the endogenous *rab11a* mRNA levels rose 2.5-fold, similar to the increase observed for *cyclinD1* (Fig. 3). Thus, the induction of c-Jun alone led to increased expression of *rab11a*, clearly suggesting that the recycling endosome gene is an AP-1 target gene in different cellular contexts.

Pharmacological inhibition of JNK leads to reduced Rab11 protein levels and TfR recycling

Constitutive AP-1 induction in *Theileria parva*-infected lymphocytes is JNK dependent (Galley *et al.*, 1997; Chaussepied *et al.*, 1998). To test the contribution of the JNK pathway to *rab11a* induction we used short-term (18 h) pharmacological inhibition of JNK by the SP600125 drug and observed a threefold reduction in *rab11a*-driven

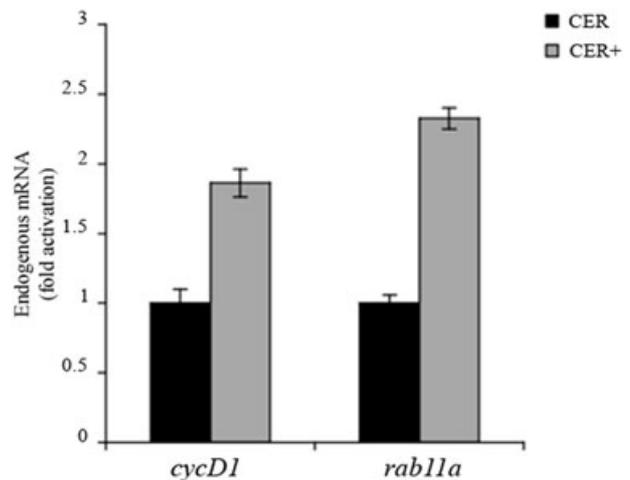


Fig. 3. Induction of CER fusion protein in NIH3T3 cells leads to an increase in the endogenous levels of *cyclinD1* and *rab11a* expression. NIH3T3-CER cells were serum starved overnight and then treated with oestrogen for 24 h. The levels of *cyclinD1* and *rab11a* mRNA were quantified using 'real-time' PCR and standardized using 18S RNA controls. The level of expression in non-induced cells was set at 1 (black columns). Induction of the CER fusion protein led to a twofold induction of *cyclinD1* and a 2.5-fold induction of *rab11a* expression. Standard deviation bars were calculated from at least three independent experiments.

luciferase activity (Fig. 4A, bottom left). Interestingly and significantly, the extent of reduced *rab11a* promoter activity was approximately that observed upon killing the parasite (Fig. 2A), and is comparable to that obtained by mutating the AP-1 binding site (Fig. 2B). JNK inhibition-induced loss of *rab11a* promoter activity was accompanied by a decrease in the levels of Rab11 protein, when compared with protein kinase B (PKB) (Fig. 4A, top) and a concomitant reduction in TfR surface expression (Fig. 4A, bottom right) in SP600125-treated cells. Notably, JNK inhibition had no effect on CD45 expression (Fig. 4A, bottom right). Thus, JNK/AP-1-mediated activation of the *rab11a* promoter correlates with an increase in Rab11 protein and heightened surface expression of TfR, most likely due to accelerated recycling of the receptor back to the surface of infected B cells that we have previously described to involve Rab11A (Baumgartner *et al.*, 2005).

siRNA knockdown of bovine *jnk1* reduces AP-1 transactivation, *rab11a* promoter activity and TfR surface expression

To confirm that the downregulation in AP-1 transactivation and TfR recycling observed upon treatment with SP600125 were specifically due to JNK inhibition, we reduced JNK levels using specific bovine *jnk1* siRNA oligonucleotides (Fig. 4B). Three independent siRNA oligonucleotides, each based on a specific bovine *jnk1* sequences (see *Experimental procedures*), reduced

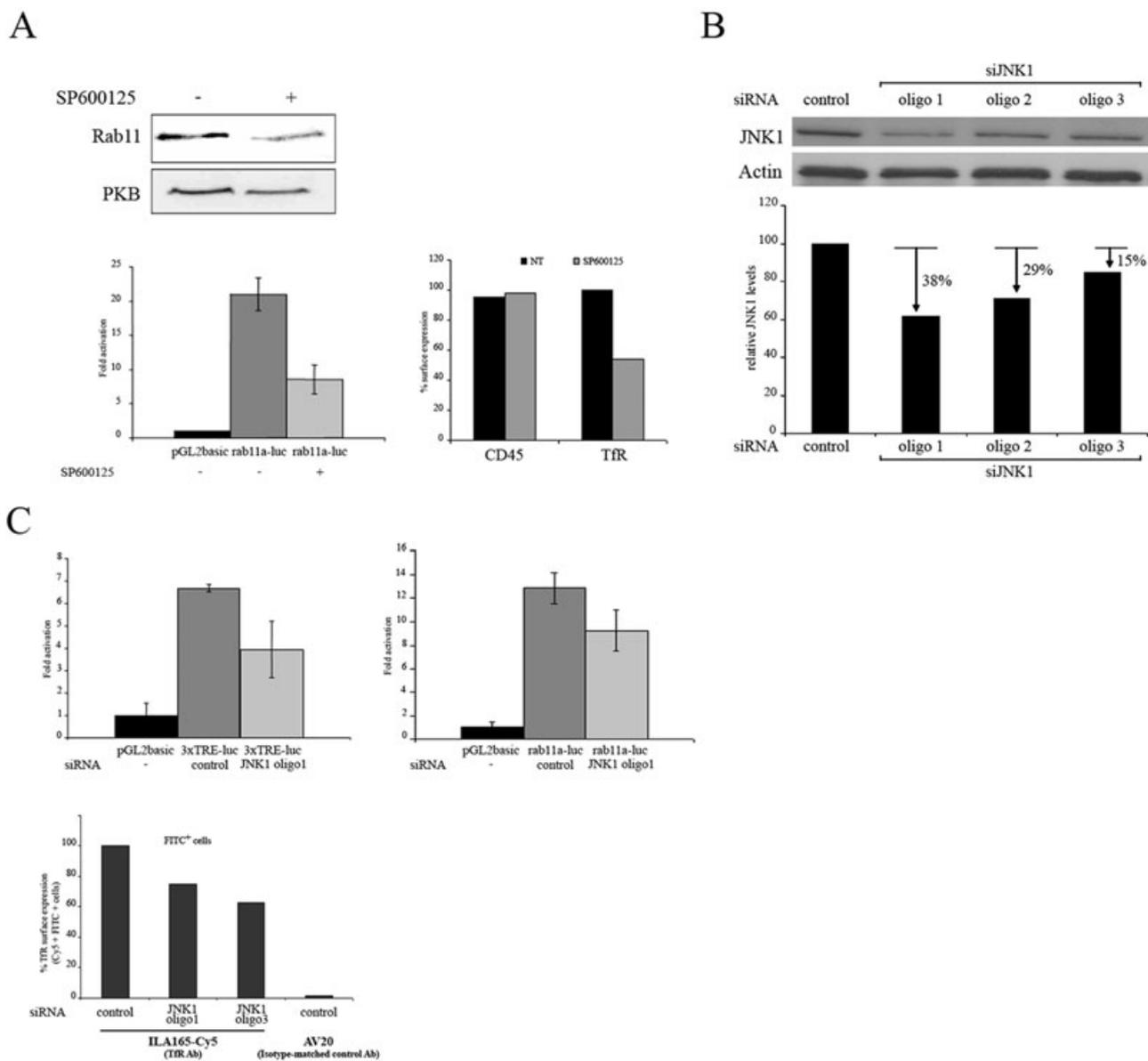


Fig. 4. AP-1 activation, Rab11 protein levels and TfR surface expression are JNK-dependent in *T. parva*-infected B cells.

A. Inhibition of JNK by SP600125 leads to diminished Rab11 protein levels compared with PKB. Top: *T. parva*-infected B cells (B2) were treated 18 h with SP600125 and the level of Rab11 protein analysed by Western blot and compared with PKB in both drug-treated and non-treated cells. Bottom: (Left) JNK inhibition by SP600125 leads to threefold loss in *rab11a* promoter activity. (Right) The level of TfR surface expression was estimated by FACS [using an IgG1 monoclonal antibody (IL-A165) to bovine TfR and a FITC-tagged secondary antibody] and compared with CD45 expression and an isotype-matched control AV20 antibody. Non-treated (NT) cells are represented by black histograms and JNK inhibition by SB600125 (grey histograms) leads to 50% reduction in TfR surface staining compared with CD45 surface expression.

B. siRNA knockdown of *jnk1* leads to loss of JNK1 protein. Infected B cells were treated with three different siRNA oligos specific for bovine *jnk1* and their effect on JNK1 protein levels were compared with that of a non-specific siRNA oligo control. siRNA *jnk1* oligo1 was the most efficacious reducing JNK levels by 38%.

C. Loss of JNK1 protein leads to reduced AP-1- and *rab11a*-driven luciferase activity and diminished TfR surface staining. Top: (Left) Infected B cells treated with siRNA *jnk1* oligo1 display reduced AP-1-driven luciferase activity. (Right) Infected B cells treated with siRNA *jnk1* oligo1 display reduced *rab11a*-driven luciferase activity. Bottom: Infected B cells treated with siRNA *jnk1* oligo1 and oligo3 express less TfR at their surface compared with siRNA control-treated cells. The degree of ablation correlates with the ability of each siRNA *jnk1* oligo to knockdown JNK1 protein levels.

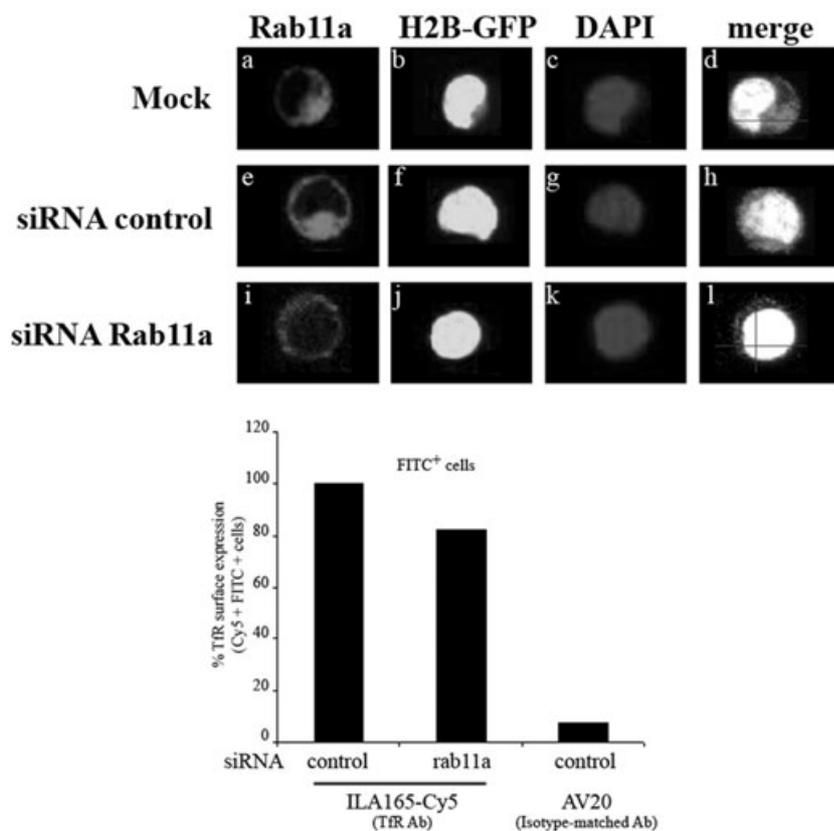


Fig. 5. siRNA knockdown of Rab11A leads to reduced surface TfR staining. Top: Bovine *rab11a*-specific siRNA oligos were transfected into infected B cells together with a plasmid encoding GFP and individual cells examined for Rab11A expression. At the single cell level Rab11A expression is reduced only in GFP-positive cells. Comparison of several individual cells gave an average reduction of 17% (not shown). Bottom: TfR surface staining is reduced in cells treated with the *rab11a*-specific siRNA oligo compared with cells treated with non-specific control siRNA oligos.

JNK1 protein levels to varying degrees; oligo1 was the most efficacious, reducing JNK1 protein levels by 38% (Fig. 4B). We therefore assayed oligo1 siRNA for its effect on AP-1 transactivation, *rab11a*-driven luciferase activity and TfR surface expression (Fig. 4C). We observed a clear reduction in both *rab11a* promoter activity and AP-1 transcriptional activity, measured by the 3XTRE reporter (Fig. 4C, top). Furthermore, the JNK1 siRNA knockdown caused a significant reduction in TfR surface expression, which correlated with the extent of efficacy of the siRNA oligonucleotides (Fig. 4C, bottom). Thus, RNA interference knockdown of bovine JNK1 phenocopied effects of the SP600125 pharmacological inhibitor, leading to a reduction in AP-1-driven transcription, *rab11a* promoter activity and TfR recycling to the B cell surface.

siRNA knockdown of bovine rab11a leads to reduced TfR recycling and surface expression

We previously reported that transient transfection of a plasmid expressing Rab11A locked in the GDP form reduced TfR surface expression due to inhibition of Rab11A-mediated endosome recycling (Baumgartner *et al.*, 2005). Here, we demonstrate that direct siRNA knockdown of the *rab11a* gene also leads to reduced Rab11A protein levels and TfR surface expression (Fig. 5,

bottom). An oligonucleotide designed specifically against the bovine *rab11a* gene led to a modest reduction in Rab11A protein levels in *Theileria*-transformed B cells as monitored by immunofluorescence (Fig. 5, left panel). The cellular consequences of inhibiting Rab11A protein expression were demonstrated by the reduction (around 20%) in recycling of TfR to the B lymphocyte cell surface. Thus, similar to expression of a dominant-negative (GDP-on) mutant of Rab11A (Baumgartner *et al.*, 2005) siRNA knockdown of Rab11A leads to reduced TfR surface expression.

Discussion

The study of *Theileria* infection of bovine lymphocytes offers a model system to explore the cellular response to an intracellular parasite, as well as the molecular mechanisms that the parasite exploits to induce lymphocyte transformation. In this study, we have identified a parasite-induced signal transduction pathway that leads to activation of the *rab11a* gene and subsequent Rab11A-mediated endosome recycling.

The Rab11A small GTPase has been shown to play an important role in mediating TfR recycling (Ullrich *et al.*, 1996; Ren *et al.*, 1998; Trischler *et al.*, 1999; Lapierre *et al.*, 2003). Thus, the elevated levels of Rab11A

associated with parasite infection might contribute to the enhanced transferrin uptake, which probably is required by proliferating *Theileria*-infected B cells (Baumgartner *et al.*, 2005). We show that *Theileria* infection of B cells is associated with increased *rab11a* promoter activity and elevated levels of Rab11 protein. We detected elevated Rab11 protein levels in *T. annulata*-infected TBL3 cells, compared with non-infected BL3 B cells (Fig. 1), and upon drug-induced parasite death *rab11a* promoter activity and Rab11A protein levels are diminished in *T. parva*-infected B cells (Fig. 2A). Furthermore, we demonstrated that *rab11a* is an AP-1 target gene and that the putative AP-1 binding site contributes to *rab11a* promoter activity in *T. parva*-infected B cells (Fig. 2B). Thus, the AP-1 transcription factor, whose induction characterizes *Theileria*-dependent transformation of bovine lymphocytes, contributes to elevated *rab11a* transcription (Fig. 2 and Chaussepied *et al.*, 1998; Baumgartner *et al.*, 2000; Baumgartner *et al.*, 2003).

Our observation (Fig. 3) that CER induction in fibroblasts led to an increase in endogenous *rab11a* mRNA confirms that this gene as a *bona fide* AP-1 target in different cellular contexts. Indeed, the *rab11a* gene is also a target of c-Fos in mouse keratinocytes and epithelial skin tumours (Gebhardt *et al.*, 2005). Thus, both c-Jun and c-Fos contribute to activating *rab11a* transcription in lymphocytes, fibroblasts and keratinocytes, suggesting that this may be a general phenomenon and common feature of the transformed phenotype. The robust (18-fold) induction of *rab11a* promoter in *Theileria*-transformed B cells is most likely due to the fact that all members of the AP-1 family (Jun, Fos and ATF2) are activated in *Theileria*-infected lymphocytes and that constitutive JNK activation in these cells contributes to enhanced AP-1-driven transcription via c-Jun phosphorylation (Chaussepied *et al.*, 1998) and ATF2 phosphorylation (Galley *et al.*, 1997). Bioinformatic analysis (not shown) suggested that additional transcription factors may also contribute to *rab11a* promoter regulation and a range of different stimuli might control the *rab11a* promoter depending on the cell type. Clearly, lymphocytes that proliferate rapidly upon cytokine stimulation can induce several transcription factors that might participate in *rab11a* expression, but in *Theileria*-transformed B cells the major transcription regulator appears to be AP-1, as mutating the single site reduces promoter activity significantly (Fig. 2A).

In *Theileria*-infected lymphocytes AP-1 induction is exclusively JNK-dependent (Chaussepied *et al.*, 1998) and this may explain why inhibition by JNK had such a profound effect of TfR surface expression (Fig. 4A, bottom right). Another non-exclusive possibility is that in *Theileria*-infected lymphocytes JNK also augments TfR recycling via direct phosphorylation of rabGDI complexed to

Rab11A, as p38-mediated phosphorylation of rabGDI complexed to Rab5 has been reported to modulate Rab5 activity (Cavalli *et al.*, 2001). In this context, the rapid drop in Rab11A protein levels detected by Western blot following JNK inhibition by SP600125 (Fig. 4A) might be partly explained by stabilization of Rab11A due to JNK-mediated phosphorylation of rabGDI.

We propose a model in which the induction of AP-1 in transformed cells could activate not only cell-cycle regulators, such as cyclin D1 (Bakiri *et al.*, 2000), but also genes involved in endosome recycling, such as *rab11a* (Fig. 3) and this may contribute to the hyper-proliferative phenotype of tumour cells. In this context, it is noteworthy that forced expression of Rab25, another Rab11 family member, markedly increased proliferation and survival of ovarian and breast cancer cells (Cheng *et al.*, 2004). Thus, in *Theileria*-infected lymphocytes and in human ovarian and breast cancer cells, augmented expression of two different members of the Rab11-family is associated with a transformed cell phenotype. Moreover, we observed that Rab11A was not only increased, but when compared with Rab2 (see Fig. 1), was also preferentially associated with the membrane fraction and consequently in an active GTP-bound form. One could surmise that a guanine nucleotide exchange factor (GEF) specific for Rab11A might be constitutively active, or upregulated in *Theileria*-transformed cells. However, testing these hypotheses awaits the identification of a GEF specific for Rab11A (Hales *et al.*, 2001).

It is noteworthy that AP-1 has been reported to regulate the *TfR* gene in several systems (Beard *et al.*, 1991). Our results on c-Jun, combined with those on c-Fos (Gebhardt *et al.*, 2005), demonstrate that AP-1 regulates *rab11a* transcription and they support a role for AP-1 in controlling endosome recycling. This dual regulation would offer a mechanism for co-ordinating the regulation of recycling endosomes with cell-cycle progression during tumorigenesis. Our study highlights how *Theileria* parasites hijack the cellular machinery to drive lymphocyte proliferation. We have exploited this model, and the use of pharmacological, or RNAi inhibition, to identify the importance of the JNK/AP-1 pathway in the transcriptional induction of the *rab11a* gene and the cellular consequences for receptor recycling in *Theileria*-transformed cells.

Experimental procedures

Parasite-infected cell lines and BW720c treatment

The TBL3 cell line was derived from *in vitro* infection of the spontaneous bovine B-lymphosarcoma cell line, BL3, with the Hissar stock of *T. annulata*. TpMD409.B2 is a *T. parva*-infected B cell line. The culture conditions and B cell characteristics of the three lines have been described (Moreau *et al.*, 1999). The

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anti-parasite drug BW720c (also known as Buparvacone, Hudson *et al.*, 1985) was used at 50 ng ml⁻¹, as described (Baumgartner *et al.*, 2000). BW720c has no effect on mammalian cells (Hudson *et al.*, 1985; Coquerelle *et al.*, 1989).

Preparation of cell lysates and subcellular fractionation

All steps were performed at 4°C and the cells were broken by repeated passages through a barrel type homogenizer (Bernitech Engineering, CA, USA). The lysate, adjusted to 0.1 M sucrose, was centrifuged at 2000 r.p.m. for 5 min to remove nuclei and unbroken cells. The post-nuclear supernatant was then centrifuged at 150 000 *g* for 1 h in a Kontron TST 55.5 rotor. High-speed supernatant (cytosol fraction) and high-speed pellet (membrane fraction) fractions were carefully resuspended in cold buffer and post-nuclear supernatant were either used immediately after 5 min boiling in Laemmli buffer, or quickly frozen in liquid nitrogen and stored at -80°C. Protein quantification was performed using the Bradford technique.

Western blotting, reagents and inhibitors

Proteins were resolved on 12% polyacrylamide-SDS gels and electro-transferred to nitrocellulose. Nitrocellulose membranes were incubated with anti-rabGDI, anti-Rab2, anti-Rab11 or anti-PKB antibodies (sc-1618 and sc-7127) diluted in blocking buffer (10 mM Tris pH 8, 150 mM NaCl, 0.1% Tween 20, 5% non-fat milk). The secondary antibody was coupled to peroxidase. When re-probing was necessary, membranes were stripped 30 min at 50°C in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7) and subsequent immuno-detection was performed as described above. Antibodies were detected using the ECL technique (Boehringer) and protein bands were quantified by scanning with the Kodak Image Station 440cf (Eastman Kodak Company, Rochester, NY). Quantification of the band intensity was performed using the Kodak Digital Science 1D image analysis software. SP600125 (Anthra[1,9-cd]pyrazol-62H-one), an anthrpyrazolone inhibitor of JNK (Sigma, # S5567) was added at 25 μM every 6 h three times (18 h).

Construction of rab11a-promoter constructs and luciferase assays

Following a computer-assisted search (<http://www.cbrc.jp/research/db/TFSEARCH.html>), where the AP-1 consensus site is defined as [G/A]-[G/C/A]-T-G-A-C-N-C-A-G-[T/A] for the presence of transcription factor binding sites in the human *rab11a* promoter region, 954 bp of human genomic DNA upstream of the *rab11a* initiating ATG were PCR amplified. All oligonucleotides used contained a 5' SmaI and a 3' MluI site to facilitate cloning into pGL2basic (Promega). To certify that only the specific AP-1 site had been correctly mutated all cloned products were sequenced on both strands.

Transient transfection experiments were carried out using a 3XTREcolluciferase reporter plasmid (Berberich *et al.*, 1994), a 'wild-type' *rab11a*-luciferase reporter plasmid and a pGL2-luc basic plasmid. Each luciferase reporter construct was transiently co-transfected into the *T. parva*-infected B cell line (TpMD409. B2), together with a plasmid encoding β-galactosidase under the

control of the EF1α promoter for normalization, as described (Baumgartner *et al.*, 2000). Luciferase activity was assayed 24 h post transfection in a microplate luminometer (Micro Lumat Plus LB96v, Berthold) using a Luciferase Assay Reagent (Promega, #E1483) and compared with that of the minimal promoter in pGL2basic.

siRNA transfection and FACS analysis

The target sequence for *jnk1* and *rab11a* are specific for both bovines and humans. All siRNA duplexes were obtained from Qiagen. The siRNA oligos sequences are as follows:

si *jnk1* bov oligo 1: 5'-CTGGTAATCGACGCATCTAAA-3'.
 si *jnk1* bov oligo 2: 5'-AAGCGTGCTTACAGAGAGCTA-3'
 si *jnk1* bov oligo 3: 5'-CAGACCTAAATATGCCGGATA-3'.
 si *rab11a*: 5'-AAGAACGATACCGCGCTATAA-3'.

The sequence of control siRNA is 5'-AATTCTCCGAACGTGTCACGT-3'.

For transfection of siRNA oligos, exponentially growing cells were washed and resuspended in RPMI medium (Gibco, Invitrogen). A total of 5 × 10⁶ cells were co-transfected with 15 μg of the green fluorescent protein (GFP) plasmid (H2B-GFP) and 250 nM of the siRNA (control or JNK1 or Rab11A-specific) by electroporation with a Gene Pulser electroporator (Bio-Rad) at 280 mV, 500 μF, two pulses.

For FACS analyse of bovine TfR and CD45 expression of siRNA-transfected cells, and SP600125-treated cells were incubated 24 h after transfection with an IgG1 monoclonal anti-bovine TfR antibody (ILA165) (Naessens *et al.*, 1996) and with the CD45 antibody (clone CC171), as described (Baumgartner *et al.*, 2005). The isotype-matched control antibody AV20 against the specific chicken B cell antigen Bu-1b has been described (Rothwell *et al.*, 1996).

Quantitative RT-PCR analysis of endogenous mouse rab11a transcripts

We analysed the expression of the murine *rab11a* gene in NIH3T3 cells expressing an inducible CER fusion protein (Bossy-Wetzel *et al.*, 1997). Cells were grown in low serum conditions and c-Jun activity was induced with oestrogen, as previously described (Bossy-Wetzel *et al.*, 1997). Cytoplasmic mRNA was isolated using an RNeasy kit (QIAGEN) and cDNA prepared using Superscript II reverse transcriptase (Invitrogen). We analysed gene expression by 'real-time' quantitative PCR using SYBR Green (Applied Biosystems) and an ABI PRISM 7000 machine (Applied Biosystems). Primers for 18s mRNA were used as a control for standardization. Oligonucleotide primers used for *rab11a* transcripts: 5'-TCCAGGTTGATGGGAAACAA and 5'-GCCCTGC TGTGTCCCATATCT and for *cyclin D1* transcripts: 5'-TGAC TGCCGAGAAGTTGTGC and 5'-CCTCGGGCCGGATAGAGTT. All quantification experiments were performed in triplicate.

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