Implication of α4 phosphoprotein and the rapamycin-sensitive mammalian target-of-rapamycin pathway in prolactin receptor signalling

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Abstract

A prolactin (PRL)-responsive 3′-end cDNA encoding rat α4 phosphoprotein was previously isolated from a rat lymphoma cDNA library. Rat α4 is a homologue of yeast Tap42 and is a component of the mammalian target-of-rapamycin (mTOR) signalling pathway that stimulates translation initiation and G1 progression in response to nutrients and growth factors. In the present study, the full-length rat α4 cDNA was obtained by 5′-RACE and the 1023 bp open reading frame predicted a 340 amino acid protein of 39·1 kDa. The α4 mRNA was expressed in quiescent PRL-dependent Nb2 lymphoma cells deprived of PRL for up to 72 h but expression was downregulated within 4 h of PRL treatment. In contrast, PRL-independent Nb2-Sp cells showed constitutive expression of α4 that was not affected by PRL. Western analysis of Nb2 cell lysates or of V5-tagged-α4 expressed in COS-1 cells detected a single immunoreactive band of ∼45 kDa. Enzymatic deglycosylation of affinity-purified 45 kDa α4 yielded the predicted 39 kDa protein. Phosphorylation of Nb2 α4 was induced by PRL or 2-*O*-tetradecanoyl-phorbol-13-acetate (TPA) and further enhanced by a combination of PRL and TPA. The Nb2 α4 associated with the catalytic subunit of protein phosphatase 2A and localized predominantly in Nb2 nuclear fractions with trace amounts in the cytosol. The immunosuppressant drug rapamycin inhibited proliferation of Nb2 cells in response to PRL or interleukin-2, but had no effect on Nb2-Sp cells. Furthermore, transient overexpression of α4 in COS-1 cells inhibited PRL stimulation of the immediate-early gene interferon regulatory factor-1 promoter activity. Therefore, PRL downregulation of α4 expression and/or PRL-inducible phosphorylation of α4 may be necessary for PRL receptor (PRLr) signalling to the interferon regulatory factor-1 promoter in the Nb2 cells and, furthermore, implicates cross-talk between the mTOR and PRLr signalling cascades during Nb2 cell mitogenesis.

Journal of Endocrinology (2002) **173,** 493–506

Introduction

Prolactin receptor (PRLr) signal transduction is mediated by the activation of several receptor-associated kinase cascades and results in the transmission of a mitogenic and/or lactogenic signal in target cells (Yu-Lee 1997, Clevenger *et al.* 1998). PRLr-associated and PRL-activated Jak2 tyrosine kinase recruits and phosphorylates members of the signal transducer and activator of transcription (Stat) family of latent cytoplasmic transcription factors (Campbell *et al.* 1994, Rui *et al.* 1994). Stat binding to the interferon γ-activated sequence mediates the mitogenic signalling of PRL to the immediate-early gene interferon regulatory factor-1 (IRF-1) in the rat PRL-dependent Nb2 lymphoma cells (Stevens *et al.* 1995, Wang *et al.* 1997). PRLr signal transduction also involves three other receptor-associated molecules, p59 fyn tyrosine kinase (Clevenger & Medaglia 1994), Raf-1 serine/threonine kinase (Clevenger *et al.* 1994) and 2′-5′oligoadenylate synthetase (OAS) (McAveney *et al.* 2000). PRL activation of the p59 fyn/Sos/Vav/Raf-1/MAP kinase cascade has been reported to stimulate Nb2 cell proliferation (Clevenger & Medaglia 1994, Clevenger *et al.* 1994) whereas OAS reduced PRL induction of IRF-1 promoter activity (McAveney *et al.* 2000). PRL has also been shown to stimulate tyrosine phosphorylation of cytoplasmic protein tyrosine phosphatase (PTP1D), and formation of a PRLr–Jak2–PTP1D complex was critical for transmission of a lactogenic signal (Ali *et al.* 1996). PRLr activation also resulted in PRL internalization

(Vincent *et al.* 1997) whereby PRL is transported across the endoplasmic reticulum and nuclear envelope in a process termed nuclear retrotranslocation (Clevenger *et al.* 1990, Rao *et al.* 1995). The peptidylprolyl isomerase cyclophilin B has been revealed as an important chaperone facilitating nuclear retrotransport of PRL (Rycyzyn *et al.* 2000).

In our studies on differential gene expresssion in the PRL-dependent Nb2 and the PRL-independent Nb2-Sp rat lymphoma cell lines, we have previously identified, by differential display and screening of an Nb2-Sp cDNA library, a number of genes which are differentially expressed in Nb2 cells (with or without acute PRL treatment for 3 h) versus Nb2-Sp cells (Too 1997, Johnson & Too 2001, Too *et al.* 2001). One of these was a PRL-responsive partial cDNA encoding the rat homologue of α4 phosphoprotein (Too 1997). The action of α4 in PRL-regulated Nb2 cell proliferation is not known. The murine α4 cDNA (Inui *et al.* 1995) and human α4 (IGBP1) gene (Onda *et al.* 1997) have also been cloned. Murine α4 was originally identified in murine lymphoid cell lines as a 52 kDa protein (p52) that co-precipitated with the Ig receptor (IgR)-related MB-1 protein (Kuwahara *et al.* 1994). A novel murine α4-b genomic clone has been shown to be selectively expressed in the testis and in the brain (Maeda *et al.* 1999).

Mammalian α4 is a homologue of yeast Tap42, an essential component of the target-of-rapamycin (TOR) kinase signalling pathway controlling translational initiation and survival in yeast cells (Di Como & Arndt 1996). Yeast Tap42 associates with the serine/threonine protein phosphatases, PP2A and Sit4. The mammalian TOR (mTOR) pathway is conserved in mammalian cells. Mouse and human α4 also associate with PP2A and/or the PP2A isoforms, PP4 and PP6 (Chen *et al.* 1998, Inui *et al.* 1998). The TOR/mTOR proteins belong to a novel family of phosphatidylinositol kinase-related kinases, which include mammalian phosphatidylinositol 3-kinase. The mTOR pathway responds to mitogens and nutrients to stimulate translation initiation and cell growth via activation of p70S6 kinase (p70S6K) and inactivation of the translational repressor 4E-BP1 (or PHAS-1), as well as through effects mediated by the α4–PP2A interaction (Thomas & Hall 1997, Cutler *et al.* 1999). mTOR phosphorylation and activation of p70S6K leads to activation of ribosomal protein S6 to promote translation of 5′-terminal oligopyrimidine tract mRNAs, ribosome biogenesis and increased capacity of the translational machinery (Jiang & Broach 1999). mTOR phosphorylation and inactivation of 4E-BP1 alleviates inhibition of the eukaryotic translational initiation factor 4E to enhance the translation of all mRNAs (Thomas & Hall 1997). The α4–PP2A interaction is believed to stimulate the initiation of translation, through effects on p70S6K and/or 4E-BP1. The augmentation of global translation results in cell growth (in size) and proliferation (progression into G1). It is believed that by utilizing the mTOR pathway to sense nutrients and mitogenic signals, mammalian cells may co-ordinate responses to mitogens relative to the nutrients available to carry out the response (Cutler *et al.* 1999). The mTOR pathway is sensitive to the anti-fungal and immunosuppressive drugs, rapamycin and FK506, which bind to a cytosolic family of FK506-binding proteins (FKBPs) with peptidylprolyl isomerase activities (Sehgal 1998). The rapamycin–FKBP complex thus formed is an active intracellular toxin which diminishes the association of α4 (or Tap42) with PP2A, thereby inhibiting protein synthesis and arresting cells in G1 (Di Como & Arndt 1996, Thomas & Hall 1997). Indeed, rapamycin is a potent inhibitor of interleukin-2 (IL-2)-induced T-cell proliferation (Morice *et al.* 1993*a*,*b*). Mutations in Tap42 have been reported to confer rapamycin resistance in yeast cells (Di Como & Arndt 1996).

The present study showed that PRL-responsive rat α4 is a glycophosphoprotein which also binds to PP2Ac. We also showed that rapamycin or transient overexpression of α4 inhibited PRL-stimulated cell proliferation and IRF-1 promoter activity, suggesting cross-talk between the mTOR and PRLr signalling cascades.

Materials and Methods

Hormones and drugs

Human PRL was a generous gift from Dr R P C Shiu (Department of Physiology, University of Manitoba, Manitoba, Canada). Human recombinant IL-2 was from Upstate Biotechnology (Lake Placid, NY, USA). Rapamycin, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) and biologically inactive isophorbol,4α,9α,12β,13α,20-pentahydroxytiglia-1,6-dien-3-one (4α-PHR) were from Sigma RBI (St Louis, MO, USA).

Antibodies and α4-antiserum

The commercial sources of antibodies used were as follows: mouse anti-Stat1α p91 (C-111), rabbit anti-transcription factor TFIIB (SI-1), goat anti-glucokinase (C-20) and the horse radish peroxidase (HRP)-conjugated-goat anti-mouse IgG or -donkey anti-goat IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); donkey anti-rabbit IgG–HRP conjugate (Amersham Pharmacia Biotechnology, Baie d'Urfe, Quebec, Canada); rabbit anti-mTOR (Upstate Biotechnology); mouse anti-PP2A catalytic subunit (PP2Ac) (Transduction Laboratories, Lexington, KY, USA), anti-phosphoserine (Sigma RBI); and anti-PRLr (Affinity Biotech. Inc, Golden, CO, USA). Based on the deduced rat α4 protein, anti-rat α4 antiserum was raised against a synthetic peptide conjugated to keyhole limpet haemocyanine (KLH) (KLH-EWDDWKDATHPRGYGNRQNMG; see Fig. 1), to

raise rabbit polyclonal antibodies to rat α4 (Research Genetics, Huntsville, AL, USA). The preimmune serum and antiserum were purified on protein A-Sepharose columns prior to use and yielded 3·2 and 5·2 mg protein/ml respectively.

Cell culture

Suspension cultures of the rat Nb2–11C (Nb2) lymphoma cell line were maintained in Fischer's medium for leukaemic cells containing 10% fetal bovine serum (FBS) as a source of lactogens and 10% lactogen-free horse serum (HS) as previously described (Too *et al.* 1987). Nb2 cells were growth-arrested at a cell density of $\sim 1{\cdot}0 \times 10^6$ cells/ml in medium containing 10% HS alone for 18–24 h. For bioassays, quiescent cells at $0{\cdot}2 \times 10^6$ cells/ml in 10% HS medium were treated with PRL (10 ng/ml), IL-2 (30 U/ml) or rapamycin and cells were counted on day 3 with a Coulter counter. For expression studies, quiescent cells at $0{\cdot}6 \times 10^6$ cells/ml were treated with PRL for the indicated times whereas controls were untreated. Nb2-Sp cells also express receptors for PRL but, unlike Nb2 cells, are PRL-independent and were maintained in 10% HS medium; cells at late-log phase were similarly reduced to a cell density of $0{\cdot}6 \times 10^6$ cells/ml (for expression studies) or $0{\cdot}2 \times 10^6$ cells/ml (for 3-day bioassays) prior to hormone or drug treatment. COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS.

Rapid amplification of 5′-cDNA ends (5′-RACE)

In an earlier study, the incomplete rat α4 3′-end cDNA (1220 bp) was identified by differential display and cloned from an Nb2-Sp cDNA library (Too 1997). In the present study, the 5′-end sequence was completed by 5′-RACE (Marathon cDNA Amplification Kit; CLONTECH Laboratories, Inc., Palo Alto, CA, USA) using the same Nb2-Sp cDNA library. A single base mutation was identified in the partial rat α4 cDNA clone that was reported earlier and the corrected sequence of the full-length rat α4 cDNA has been updated in GenBank (accession number AF000577).

Generation of full-length α4 cDNA (pcDNA3·1-α4-V5 clone)

The full-length α4 cDNA, with engineered 5′ *Kpn*1 and 3′ *Apa*1 sites, was obtained by two rounds of PCR of the Nb2-Sp cDNA library with the primers 5′-atggcagcg tctgaagaagagttactgctg-3′ and 5′-caaggggccctttgacactatca aatgc-3′, and 5′-gcttggtaccaagatggcagcgtctgaag-3′ and 5′-caaggggccctttgacactatcaaatgc-3′ respectively. The PCR product from the second round of PCR was restriction digested with *Kpn*1 and *Apa*1, then ligated into the *Kpn*1/*Apa*1 multiple cloning sites of the pcDNA3·1 expression vector which has a 3′-end V5 epitope (Invitrogen Corporation, Carlsbad, CA, USA).

Northern analysis and semi-quantitative RT-PCR

DNA-free total RNAs were extracted from Nb2 cells and used for Northern or RT-PCR analysis. The α4 mRNA-specific primers 5′-gagttactgctgccgcggcttccggagc-3′ and 5′-cgagcccgctgcaaatgatctagacgc-3′ gave a 304 bp product. The 18S primers used (315 bp product) were from the QuantumRNA 18S Internal Standards Kit (Ambion Inc., Austin, TX, USA). All PCR products were obtained within the linear range of the reaction.

Glutathione S-transferase (GST)-α4-V5 fusion protein

*BamH*1 and *EcoR*1 restriction sites were incorporated into the pcDNA3·1-α4-V5 clone by PCR using primers 5′-gcttggatccaagatggcagcgtctgaag-3′ and 5′-gaccggaattcg tagaatcgagaccgag-3′ to generate a 1204 bp product. This was cloned in-frame into the *BamH*1 and *EcoR*1 sites of pGEX-2T GST gene fusion vector (Amersham). Transformation of competent *E. coli* with pGEX2T-α4, induction of GST-α4-V5 expression with 100 mM isopropyl-1-thio-β-D-galactoside (IPTG) and analysis of transformants were performed as described (Ausubel *et al.* 1994). For GST-fusion protein pull-down assays, total cell lysates were incubated with GST-α4 fusion proteins conjugated to glutathione-Sepharose beads. Protein complexes were washed, resolved by SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting.

Subcellular fractionation, immunoprecipitation, affinity purification and Western analysis

Subcellular fractionation was performed as previously described (Dodd *et al.* 2000) to obtain nuclear (800 ***g*** pellet, 5 min), microsomal (100 000 ***g*** pellet, 60 min) and cytosolic (100 000 ***g*** supernatant) fractions. Proteins (8–20 μg/lane), representing about 4% of total protein from each fraction, were electrophoresed on 10% SDS-PAGE gels. Immunoprecipitation and Western analysis were performed as described (Dodd *et al.* 2000). Nb2 α4 was affinity purified using α4-antibodies bound to Plus Immobilized Protein G (Pierce, Rockford, IL, USA). Affinity-purified α4 was deglycosylated with an Enzymatic Deglycosylation Kit (BioRad Laboratories Ltd, Mississauga, Ontario, Canada) following the manufacturer's instructions.

[^{32}P]-orthophosphate labelling and phosphoamino acid analysis

Nb2 cells were labelled with [^{32}P]-orthophosphate (TPA ± PRL) as described (Rayhel *et al.* 1988, Fulton *et al.* 1999), with modifications. Briefly, quiescent Nb2 cells (10×10^6 per treatment) were pelleted at 200 ***g*** for

5 min at room temperature, washed twice with phosphate-free RPMI 1640 (Life Technologies, Burlington, Ontario, Canada) and resuspended in 5 ml of the same medium for a 1-h incubation at 37 °C. Cells were incubated with [^{32}P]-orthophosphate (0·1 mCi/ml) for 70 min at 37 °C. TPA (20–80 nM) ± PRL (10 ng/ml) was added and inactive phorbol 4α-PHR (40 nM) was used as negative control. After 20 min at 37 °C, the cells were pelleted at 200 ***g*** (5 min), washed twice with ice-cold phosphate-buffered saline (PBS) containing 10 μM Na_3VO_4, 10 mM NaF, 10 mM $Na_4P_2O_7$ and 440 μM EDTA. Cell pellets were resuspended in 0·5 ml RIPA buffer and total cell lysates were used for immunoprecipitation with anti-α4 antibodies. For phosphoamino analysis, immunoprecipitated [^{32}P]-labelled proteins were resolved by SDS-PAGE, excised from the gel, washed twice with 1 ml 25% isopropanol (v/v)–10% methanol (v/v) and minced in 150–200 μl 50 mM ammonium bicarbonate. Trypsin-L-[1-tosylamido-2-phenyl] ethyl chloromethyl ketone (10 μl of a 10 mg/ml stock) was added to each tube for an overnight digestion at 37 °C. Insoluble material was precipitated and the supernatants were lyophilized and redissolved in 50 μl 6 M HCl for hydrolysis at 110 °C for 90 min. Dried samples and phosphoamino acid standards were dissolved in 10 μl distilled water for thin-layer chromatography in isopropanol:H_2O:HCl (65:8·4:6·6). Samples were visualized with ninhydrin (0·5% in acetone (w/v)) or by autoradiography.

Chloramphenicol acetyltransferase (CAT) assay

Transient transfection of COS-1 cells and CAT assays were performed as described (McAveney *et al.* 2000). CAT activity was determined by the fluor-diffusion method (Neumann *et al.* 1987) with slight modifications. Briefly, COS-1 cells (2×10^5 per well in a 6-well dish) were cultured overnight in DMEM–10% FBS and then rinsed with DMEM. Transient transfection was performed with LipofectAMINE-DNA mixture containing 5 μl LipofectAMINE (Life Technologies, Grand Island, NY, USA), 1 μg pECE-Nb2-PRLr, 0·2 μg 1·7 kb IRF-1-CAT, 1 μg pcDNA3-Stat1α and 1 μg pcDNA3-α4 or 1 μg pcDNA3-antisense α4 or 1 μg control pcDNA3·1, in a total volume of 1 ml DMEM. The antisense pcDNA3-α4 construct was made by restriction digestion of pcDNA3·1-α4-V5 to obtain a 252 bp *Kpn*1/*Kpn*1 insert containing the initiation factor ATG and a 6·5 kb DNA fragment; these were religated and constructs with sense or antisense orientations were determined by restriction digestion. After 5 h at 37 °C, the LipofectAMINE-DNA mixture was aspirated and replaced with DMEM–1% HS for 18–24 h, then washed twice with PBS and harvested in PBS containing 1 M EDTA. The cells were pelleted by centrifugation at 800 ***g*** for 5 min at 4 °C followed by resuspension in 100 μl 100 mM Tris, pH 7·8 containing 0·1% Triton X-100. After 10–15 min on ice, nuclei and cell debris were pelleted by centrifugation (800 ***g***, 15 min) and the supernatant recovered. Aliquots were used for BioRad protein assay. To assay for CAT activity, equal amounts of protein extracts were mixed with 100 mM Tris, pH 7·8 containing 0·1% Triton X-100 in a total volume of 200 μl and placed in 7 ml scintillation vials. The vials were heated at 70 °C for 10 min to inactivate heat-labile cellular acetyltransferases and then cooled at room temperature for 10 min before the addition of 75 μl reaction cocktail containing 0·5 M Tris–HCl, pH 7·8, 0·2 mCi [^{3}H]-acetyl CoenzymeA and 1·75 mM chloramphenicol. ScintiLene SX2–4 (Fisher Scientific, Nepean, Ontario, Canada) was pre-warmed to 37 °C and 3 ml was carefully layered over the aqueous samples. The vials were capped and samples were counted for five cycles for at least 1 min/sample with no interruptions. The data were analyzed by regression analysis and expressed as c.p.m./μg protein.

Statistical analysis

Analysis of variance and Scheffe's F-test were performed using Abacus Concepts, Statview (Abacus Concepts, Inc., Berkeley, CA, USA; 1992).

Results

Full-length sequence of rat α4 cDNA by 5′-RACE

A partial rat α4 3′-end cDNA was identified as PRL-responsive in the Nb2 cells (Too 1997). The full-length rat α4 cDNA (1239 bp; GenBank accession number AF000577) was obtained by 5′-RACE and it has an open reading frame of 1023 bases encoding a 340 residue protein with a predicted molecular weight (MW) of 39·1 kDa. The deduced rat α4 protein has significant sequence identity with α4 and α4-related molecules of mouse (α4, 97%; α4-b, 85%), human (α4/IGBP1, 95%) and yeast (Tap42, 48%) (Fig. 1; see also Maeda *et al.* 1999). Like other α4 molecules, rat α4 has multiple potential phosphorylation sites for protein kinase C (PKC) or casein kinase 2, sites for N-linked glycosylation and potential myristylation as well as a potential SH3-binding motif.

α4 mRNA is widely expressed in rat and human tissues or cell lines

The α4 transcript was expressed in quiescent Nb2 cells deprived of PRL for 20 h (Fig. 2A and B, right-hand panel; 0 h time) but this level of expression decreased within 4 h of PRL treatment as shown by Northern (Fig. 2A, 1·4 kb mRNA) or RT-PCR analyses (Fig. 2B, 304 bp product). Nb2 expression of α4 during PRL deprivation was maintained for as long as 72 h (Fig. 2B, left-hand panel). The PRL-independent Nb2-Sp cells have receptors for PRL, but PRL had no detectable effect on α4 mRNA levels in these cells (Fig. 2C).

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rα4      MAASEEELLL PRLPELFETS KKLLEELEVA TEPTGSRTIQ DKVSKGLELL EKAAGMLSQL DLFSRNEDLE 70
mα4      MAASEDELLL PRLPELFETS KKLLEDVEVA TEPTGSRTIQ DKVSKGLELL EKAAGMLSQL DLFSRNEDLE 70
mα4-b    MASFTEEMQK PKLRELLETG IQLLEEVEAA TQPTGSKPIQ EKVREALKLL EKASDMLSQL DLFSRNEDWE 70
hα4      MAA-EDELQL PRLPELFETG RQLLDEVEVA TEPAGSRIVQ EKVFKGLDLL EKAAEMLSQL DLFSRNEDLE 69
yTap42   MASVTEQFN- -DIISLYSTK ---LEHTSLR QDSPEYQGLL LSTIK--KLL NLKTAIFDRL ALFSTNETID 63

rα4      EIASIDLKYL MVPALQGALT MKQ------V NP--SKRLDH LQRAREHFIH FLTQCHCYHV AEFQLPQTKN 132
mα4      EIASTDLKYL MVPALQGALT MKQ------V NP--SKRLDH LQRAREHFVH FLTQCHCYHV AEFQLPQTKT 132
mα4-b    EIASADLKYL MLPALKGALT LKL------V GS--SKRLGL LQDAREHFMN FLTQTHSYHV ADFQLPWAQS 132
hα4      EIASTDLKYL LVPAFQGALT MKQ------V NP--SKRLDH LQRAREHFIN YLTQCHCYHV AEFELPKTMN 131
yTap42   DVSTASIKFL AVDYYLGLLI SRRQSNDSDV AQRQSMKLIY LKKSVESFIN FLTLLQDYKL LDPLVGEKLG 133

rα4      NSAENNTARS SMAYP----- -NLVAMASQR QAKIERYKQK KEVEHRLSAL KSAVESGQAD DER---VREY 193
mα4      NSAENNTASS SMAYP----- -NLVAMASQR QAKIERYKQK KEVEHRLSAL KSAVESGQAD DER---VREY 193
mα4-b    SSMEGNPAAT SDAQEQ---- -NLVAMASQR QTKIQRYKQK KAVEQRLSSL KSAVESGQAD DER---VREY 194
hα4      NSAENHTANS SMAYP----- -SLVAMASQR QAKIQRYKQK KELEHRLSAM KSAVESGQAD DER---VREY 192
yTap42   NFKDRYNPQL SELYAQPKNN KDLSGAQLKR KEKIELFQRN KEISTKLHCL ELELKNNDED HDHDELLREL 203

rα4      YLLHLRRWIG ISLEEIESID QEI---KILK D--KDSPREE SACQSSLPEK PP---MKPFI LTRNKAQAKV 255
mα4      HLLHLRRWIA VSLEELESID QEI---KILK E--KDSPREE TACHSSLPEK PP---MKPFI LTRNKAQAKV 255
mα4-b    YLLQLRRWIS ISLDEIENIE QEI---EILR E--RDSLGET SASRSSPQER PP---MKPFI LTRNKAQAKV 256
hα4      YLLHLQRWID ISLEEIESID QEI---KILR E--RDSSREA STSNSSRQER PP---VKPFI LTRNMAQAKV 254
yTap42   YLMRLHH--- FSLDTINNIE QNLFECEMLS NFLKNSVHEV KSSGTQIRKE SNDDDSTGFT DKLENINKPL 270

rα4      FGTGYPSLAT MTVSDWYEQH QKYGA----- ----LPDRGI AKPPSADFQR AAQQQEDQEQ KDEEN--EEK 314
mα4      FGTGYPSLAT MTVSDWYEQH QKYGV----- ----LPDRGI AKPASADFQR AAQQQEDQEQ KDEES--EEK 314
mα4-b    FGTGYPSLAT MTVSDWYEQR QKNEV----- -SPTLQEAEK QAPPSETFTV SEKEEPDLEQ KEDE---DEN 317
hα4      FGAGYPSLPT MTVSDWYEQH RKYGA----- ----LPDQGI AKAAPEEFRK AAQQQEEQEE KEEED--DEQ 313
yTap42   IDKKGQVLRN FTLVDKRQQL QQKVRGYGQY GPTMSVEEFL DKEFEEGRVL QGGEEPEQAP DEENMDWQDR 340

rα4      ALHRMREWDD WKDTHPRGYG NRQNMG  340
mα4      ALHRMREWDD WKDTHPRGYG NRQNMG  340
mα4-b    ALHRMQEWDD WKDTHPRGYG NRQNMG  343
hα4      TLHRAREWDD WKDTHPRGYG NRQNMG  339
yTap42   ETYKAREWDE FKESHAKGSG NTMNRG  366
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Figure 1 Alignment of deduced α4 and related molecules. Rat α4 (rα4; GenBank accession number AF000577), murine α4 (mα4; L31652), murine α4-b (mα4-b; AJ010637), human α4 (hα4; Y08915) and yeast Tap42 (yTap42; U43890) were aligned by multiple sequence alignment. (Thin overline) PP2Ac-binding region; (broken overline) proline-rich SH3 binding-like motif; (bold overline) C-terminus conserved motif (anti-α4 antibodies were raised to this motif); (hatched underline) potential myristylation site. Amino acid numbers are denoted on the right.

Northern analysis showed ubiquitous expression of a single α4 transcript (1·4 kb) in the rat tissues examined, with the exception of the testes which did not express α4 (Fig. 3). An α4-b isoform has been shown to be selectively expressed in murine testes and brain (Maeda *et al.* 1999); thus, the rat testes may express an α4-b homologue. The Nb2 cells are lymphoid tumour cells and Northern analysis of commercial poly(A)$^+$ blots showed a 1·4 kb α4 transcript in all the human immune tissues and cancer cell lines examined (Fig. 4).

α4 is glycosylated (45 kDa) and predominantly nuclear

Rat α4 has a predicted MW of 39·1 kDa. When rat α4 was expressed in *E. coli* as a GST-α4 (V5-tagged) fusion protein, a protein band of about 65 kDa was induced by IPTG (Fig. 5A). Thrombin cleavage released α4 (~38 kDa) from the GST moiety (27 kDa) (Fig. 5A). The rat V5-tagged α4 was expressed in COS-1 cells and Western analysis with anti-V5 antibodies detected a single immunoreactive band of ~46 kDa (Fig. 5B), indicating that the α4 protein (45 kDa; the V5 epitope is 1·4 kDa) undergoes post-translational modification in eukaryotic cells.

Rat α4 is a component of the mTOR pathway and mTOR (240 kDa) was detected in Nb2 and Nb2-Sp cells (Fig. 6A). Subcellular fractionation and Western analysis of quiescent Nb2 cell fractions with α4-specific antibodies showed a single immunoreactive band of 45 kDa (Fig. 6B) which was not detected by preimmune serum (Fig. 6C). The Nb2 α4 localized predominantly in the nuclear fraction with trace amounts in the cytosol; the purity of the

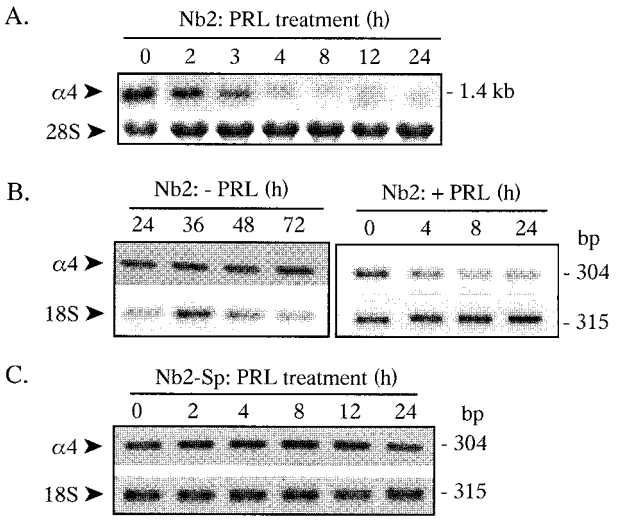

Figure 2 Prolactin (PRL) decreases α4 expression in Nb2, but not Nb2-Sp cells. Nb2 cells were growth-arrested for 20 h (− PRL, 0 h controls) and then treated with PRL (10 ng/ml) for the indicated times. Total RNA was extracted for (A) Northern blotting (1·4 kb α4 mRNA) or (B, right-hand panel) RT-PCR (304 bp α4-specific product). (B, left-hand panel) RT-PCR analysis of Nb2 cells deprived of PRL for the indicated times. (C) RT-PCR analysis of Nb2-Sp cells treated with PRL (10 ng/ml). Ethidium bromide-stained 28S or the 18S rRNA-specific PCR product (315 bp) were used as internal standards. All PCR reactions were in the linear range of amplification. Each set of data is representative of two to three independent and reproducible experiments.

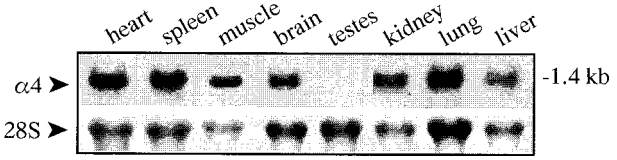

Figure 3 Tissue expression of α4. Northern analysis (25 µg total RNA/ml) was performed with tissues from male rats. Ethidium bromide-stained 28S rRNA was used as a loading control. Representative of tissue samples from two male rats.

fractions was confirmed by specific localization of the transcription factor TFIIB in the nucleus, glucokinase in the cytosol and PRLr in the membrane and cytosol (Fig. 6B). Human and murine α4 have been reported to associate with the catalytic subunit PP2A (see below) and PP2Ac was also found predominantly in the nuclear fraction with trace amounts in the microsomal and cytosolic fractions (Fig. 6B). Affinity-purified α4, after enzymatic deglycosylation, revealed the predicted 39 kDa protein (Fig. 6D). Furthermore, PRL downregulation of the α4 mRNA in Nb2 cells was accompanied by a decrease of the α4 protein, of about twofold, which was detectable after 8–24 h of PRL treatment (Fig. 6E) but not earlier at 4 h (data not shown).

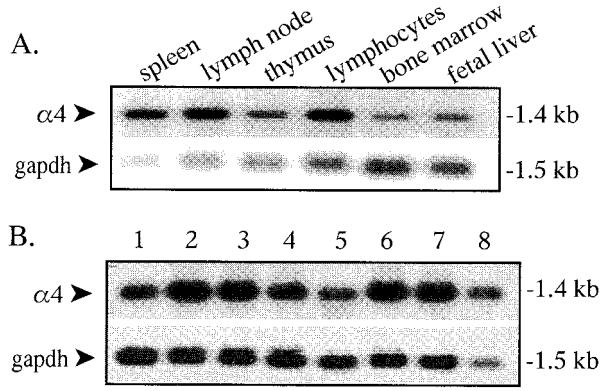

Figure 4 Human immune tissues and cancer cell lines express α4. Commercial poly(A)$^+$ Northern blots of (A) human immune tissues or (B) cancer cell lines. Glyceraldehyde-3-phosphate dehydrogenase (gapdh) (1·5 kb mRNA) was used as a loading control. In (B), lane 1, HL-60 promyelocytic leukaemia; lane 2, HeLa ovarian carcinoma; lane 3, K562 chronic myelogenous leukaemia; lane 4, MOLT-4 lymphoblastic leukaemia; lane 5, RAJI Burkitt's lymphoma; lane 6, SW480 colorectal adenocarcinoma; lane 7, A549 lung carcinoma; and lane 8, G361 melanoma.

Rat α4 interacts with PP2Ac

Human and murine α4/α4-b bind specifically to PP2Ac (Murata *et al.* 1997, Chen *et al.* 1998, Inui *et al.* 1998, Maeda *et al.* 1999). Immunoprecipitation studies showed that Nb2 α4 interacted with PP2Ac (Fig. 7A). Pull-down assays of Nb2 cell lysates with immobilized GST-α4 fusion protein also revealed an interaction of the GST-α4 with PP2Ac (Fig. 7B). The α4–PP2Ac interaction was seen for as long as 8 h after PRL treatment (data not shown).

TPA and PRL induce phosphorylation of α4

The α4 proteins have multiple potential phosphorylation sites for the serine/threonine kinase, PKC. Treatment with the tumour promoter TPA (activator of PKC) for 20 min has been shown to stimulate maximal phosphorylation of murine α4 and of several other proteins immunoprecipitated by anti-α4 antibodies (Kuwahara *et al.* 1994). In our study, Nb2 cells were radiolabelled with [^{32}P]-orthophosphate (± TPA or PRL) and α4 immunoprecipitates were analyzed for inducible phosphorylation of α4 and/or α4-associated proteins. PRL alone stimulated phosphorylation of at least three immunoprecipitated bands of 45 kDa (α4), 50 kDa and 120 kDa as compared with controls (Fig. 8A). Samples treated with TPA alone also showed phosphorylation of these three bands and a 25 kDa protein while the inactive phorbol 4α-PHR alone had no effect (data not shown). PRL and TPA (20–40 nM) in combination enhanced inducible phosphorylation of 120, 50, 45 and 36 kDa proteins (Fig. 8A and B). Thus, using both PRL and TPA (40 nM), these four [^{32}P]-immunoprecipitated bands from triplicate samples

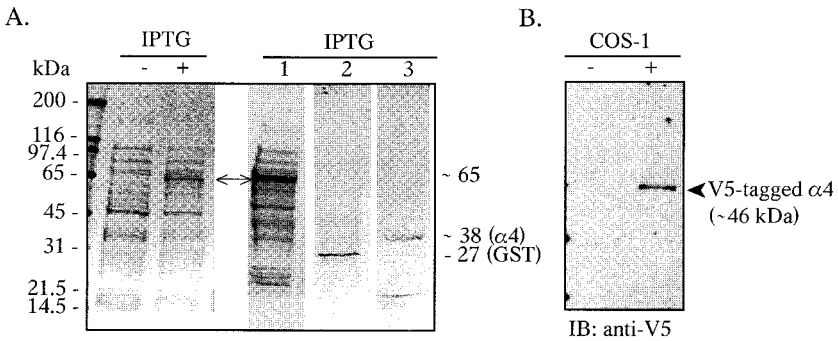

Figure 5 Eukaryotic α4 is 45 kDa. (A) The full length α4-V5-tagged cDNA was cloned into pGEX2T as described in Materials and Methods. GST-α4-V5 expression in *E. coli* was induced with 100 mM IPTG (+) while controls were untreated (−). An IPTG-inducible band of ~65 kDa was obtained (arrows). Thrombin cleavage of the 65 kDa protein (lane 1) released the 27 kDa GST moiety (lane 2) and the 38 kDa recombinant α4 (lane 3) as visualized with Coomassie Brilliant Blue R-250. (B) COS-1 cells were transfected with full-length α4 cDNA cloned into the pcDNA3·1-V5-tagged expression vector (+) and compared with untransfected controls (−). Cell lysates were used for Western blotting with anti-V5 antibody.

were excised from the gels and individually pooled. Phosphoamino acid analysis showed that the 120, 50, 45 and 36 kDa bands were phosphorylated predominantly on serine/threonine residues (Fig. 8C, lanes 1–4). The 25 kDa band that was phosphorylated by TPA alone (or TPA+PRL) was also analysed and was phosphorylated on serine, threonine and tyrosine residues (Fig. 8C, lane 5). The dark radioactive band in the solvent front of each sample was likely due to complete sample hydrolysis. Thus, PRL+TPA in combination stimulated serine/threonine phosphorylation of α4 (45 kDa) and of at least three α4-associated proteins of 36, 50 and 120 kDa. The 36 kDa phosphoserine/threonine band may or may not be PP2Ac since PP2Ac was shown to be phosphorylated on tyrosine residues by serum stimulation (Chen *et al.* 1994). PRL-/TPA-induced serine phosphorylation of α4 was confirmed by immunoprecipitation with anti-α4 antibodies followed by immunoblotting with anti-phosphoserine antibodies (Fig. 8D).

Rapamycin inhibits Nb2 cell proliferation

The α4 protein is a component of the rapamycin-sensitive mTOR pathway and the effects of rapamycin on Nb2 cell proliferation in response to PRL or IL-2, which also acts as a mitogen in these cells, was investigated. PRL- or IL-2-stimulated Nb2 cell proliferation was maximally inhibited by 100 nM rapamycin (Fig. 9A). Rapamycin, at a concentration of 100 nM, inhibited Nb2 cell proliferation in response to increasing concentrations of PRL or IL-2 by about 50% (Fig. 9B and C). Surprisingly, Nb2-Sp cell proliferation was not affected by rapamycin at concentrations as high as 10 μM (Fig. 9D).

α4 overexpression inhibits PRL signalling to the IRF-1 gene

Nb2 sensitivity to rapamycin indicates a functional mTOR signalling pathway. The possibility of an effect of the mTOR pathway on PRLr action was examined by ectopic overexpression of α4 in COS-1 cells. Control COS-1 cells expressed α4 and Stat1α proteins (Fig. 10A, lane 1). Endogenous levels of Stat1α were increased about threefold by transient transfection of the Stat1α construct (Fig. 10A, lanes 2 and 3). When co-transfected with the α4 construct, COS-1 cells showed a fivefold increase of endogenous α4 without any adverse effect on Stat1α protein synthesis (Fig. 10A, lane 4). The antisense-α4 construct used did not obliterate endogenous levels of α4 but was used as an additional control. CAT assays indicated that overexpression of α4 consistently decreased basal IRF-1 promoter activity by 30–40% and PRL-stimulated IRF-1 promoter activity by 40–50% (Fig. 10B). Studies reporting OAS inhibition of IRF-1 promoter activity also showed inhibition of both basal and PRL-inducible IRF-1 promoter activities (McAveney *et al.* 2000). Taken together, the present data suggest that ectopic overexpression of α4 inhibits PRLr signalling to the IRF-1 promoter.

Discussion

The present study showed that PRL downregulated Nb2 α4 mRNA expression which was accompanied by a modest decrease in α4 protein levels. PRL also induced phosphorylation of the Nb2 α4 protein, an effect that was enhanced by a combination of PRL and TPA. The α4 is a glycoprotein and was predominantly nuclear. Similar to

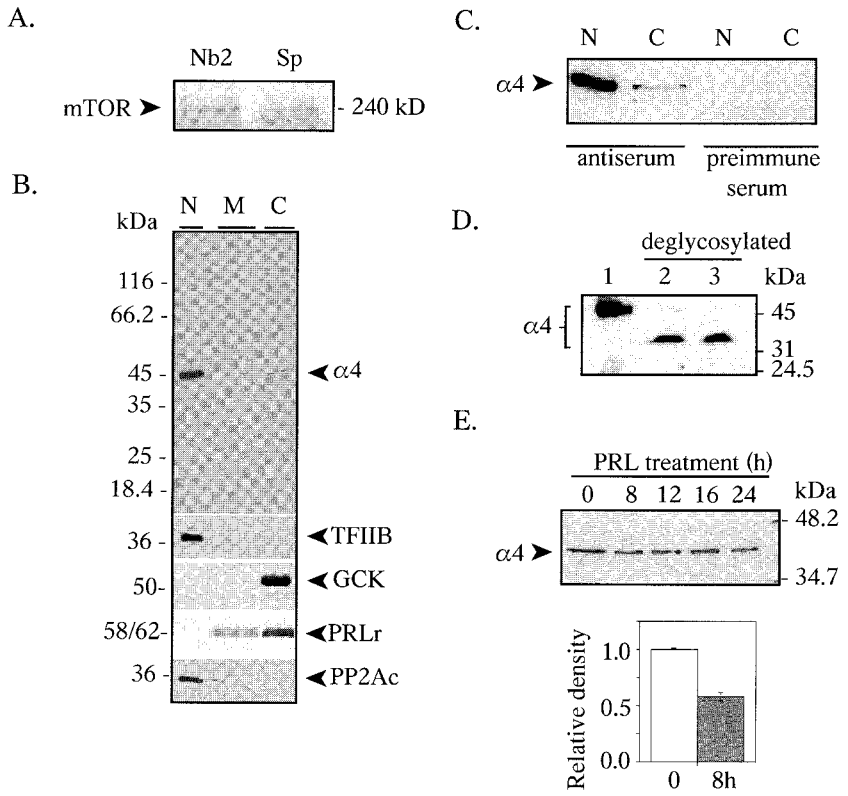

Figure 6 Nb2 α4 is glycosylated and predominantly nuclear. (A) Western analysis of immunoprecipitated mTOR in Nb2 and Nb2-Sp cell lysates. (B) Nuclear (N; 800 ***g*** pellet), microsomal (M; 100 000 ***g*** pellet) or cytosolic (C; 100 000 ***g*** supernatant) fractions from quiescent Nb2 cells were used for Western analysis of α4 (8–20 μg protein/lane; representing 4% of total protein in each fraction). Protein markers used were: TFIIB, glucokinase (GCK) and PRLr. (C) Nuclear (N; 800 ***g*** pellet) and cytosolic (C; 800 ***g*** supernatant) fractions from Nb2 cell lysates were Western blotted (20 μg protein/lane) with α4 antiserum or preimmune serum. (D) Affinity-purified Nb2 α4 (lane 1) was deglycosylated under denaturing (lane 2) or non-denaturing (lane 3) conditions and Western blotted with anti-α4 antibodies. In (A–D), each set of data is representative of two to three independent experiments. (E) Upper: Western analysis of α4 in arrested (0 h) or PRL-treated Nb2 cell lysates (40 μg protein/lane); lower: densitometric analysis of the 0 and 8 h time-points ($n=6$).

human and murine α4/α4-b proteins, the rat (Nb2) α4 bound specifically to PP2Ac and to other proteins which are as yet unidentified.

Murine α4 was initially found as a surface protein by external biotinylation (Kuwahara *et al.* 1994). However, murine α4 (Inui *et al.* 1995) and rat α4 (present study) have no apparent transmembrane domain or a leader sequence. The mammalian α4 proteins have a C-terminus myristylation-like site (GNRQNMG) (see Fig. 1). This motif, when located at the N-terminus, was reported to be commonly used by membrane-attached molecules involved in signal transduction (Resh 1994). Thus, it has been suggested that this C-terminus motif of α4 may be used for dynamic movement in signal transduction and not for direct membrane attachment (Inui *et al.* 1995). Our study showed cytosolic α4 in Nb2 cells as anticipated but, surprisingly, the α4 protein was predominantly in the cell nuclei. We also showed that the Nb2 α4 was an *O*-/*N*-linked glycoprotein, without an apparent leader sequence. Protein glycosylation is classically viewed to occur exclusively on extracellular and luminal polypeptides. However, *O*-glycosylation at specific serine or threonine residues by single β-*N*-acetylglucosamine moieties (*O*-GlcNAc) has been reported in many eukaryotic nuclear and cytoplasmic proteins (Hart 1997, Comer & Hart 1999). The enzymes for the attachment or removal of the *O*-GlcNAc moieties are cytosolic and/or nuclear (Comer & Hart 2000). *O*-GlcNAc modification of proteins has been implicated in nuclear transport (Miller & Hanover 1994) and has been suggested to act as an alternative nuclear transport signal on some proteins (Hubert *et al.* 1989, Duverger *et al.* 1996). Thus, it is possible that cytosolic *O*-GlcNAc

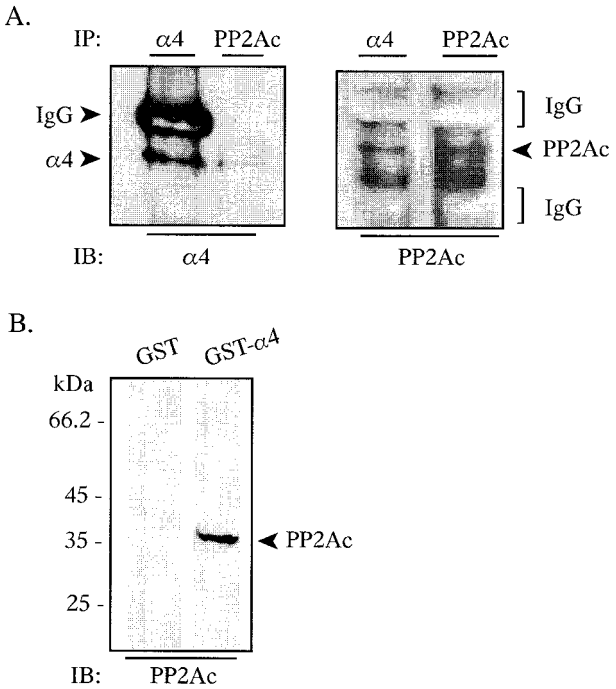

Figure 7 α4 interacts with PP2Ac. (A) Nb2 cell lysates were immunoprecipitated (IP) with anti-α4 or anti-PP2Ac and immunocomplexes were resolved by SDS-PAGE. Immunoblotting (IB) with anti-α4 or PP2Ac was performed. (B) Nb2 cell lysates were incubated with GST or GST–α4 fusion proteins conjugated to glutathione-Sepharose beads, precipitates were separated by SDS-PAGE and immunoblotted with anti-PP2Ac antibody.

modification of the Nb2 α4 phosphoprotein contributed to its nuclear localization. Interestingly, *O*-GlcNAc modification has also been implicated in signal transduction cascades (Wells *et al.* 2001) and to have functional roles in gene expression at the level of transcription and translation (Comer & Hart 1999). All known *O*-GlcNAc proteins identified to date occur as phosphorylated proteins and, in several cases, phosphorylation and *O*-GlcNAc modification are reciprocal, occurring at the same or adjacent hydroxyl moieties (Comer & Hart 2000).

It is also possible that PP2Ac (and other α4-associated molecules) act as chaperone(s) to target α4 into the nucleus. PP2Ac has been shown to undergo differential methylation, conformational changes and redistribution in the nucleus versus cytoplasm during progression into the cell cycle (Turowski *et al.* 1995). Particulate distribution of PP2A has also been reported (Sim *et al.* 1994, 1998). Nuclear PP2A has been shown to dephosphorylate the protein kinase A-phosphorylated cAMP-regulatory element binding protein (CREB) to regulate CREB transcriptional stimulation (Wadzinski *et al.* 1993), to inhibit telomerase activity in human breast cancer cells (Li *et al.* 1997) and to inhibit transcriptional activation of c-Jun and AP-1 (Al-Murrani *et al.* 1999). Conversely, it is conceivable that α4 itself (with *O*-glycosylation) may act as a chaperone targeting the phosphatase activity of PP2Ac into the cell nucleus. This is analogous to the nuclear translocation of PRL through its interaction with cyclophilin B and where PRL was viewed as a scaffold protein targeting the peptidylprolyl isomerase activity of cyclophilin B to transcription factors (Rycyzyn *et al.* 2000).

Murine α4 was initially detected as a protein that was inducibly phosphorylated by TPA (Kuwahara *et al.* 1994) and phosphorylation was exclusively on serine residues (Murata *et al.* 1997). In our study, phosphorylation of the rat (Nb2) α4 on serine/threonine residues was induced by PRL or TPA and further enhanced by PRL+TPA. The phosphorylated proteins of 36, 50 and 120 kDa may be associated with α4 or its protein partner PP2Ac. Increased phosphorylation of α4 by PRL and TPA has additional significance in Nb2 cells. TPA is known to enhance PRL-stimulated Nb2 c-*myc* expression, DNA synthesis and cell proliferation but TPA alone has no effect (Gertler *et al.* 1985, Murphy *et al.* 1988). The effector of this TPA (and PRL) action is not known but the present data suggest that it may be α4. Thus, PRL downregulation of the α4 mRNA and protein, albeit not more than twofold, was accompanied by PRL-inducible phosphorylation of α4. These events, in combination, may act to regulate efficiently the action of α4 in the proliferation of the Nb2 cells.

The immunosuppressant rapamycin is a potent inhibitor of IL-2-induced T-cell proliferation and it also inhibited growth of IL-2-dependent murine cytotoxic T-cell line, CTLL-2, by 50–70% via inhibition of G- to S-phase progression (Morice *et al.* 1993*a*,*b*). Our study showed that rapamycin partially inhibited (∼50%) Nb2 cell proliferation in response to optimal doses of PRL or IL-2, but the drug had no effect on the proliferation of the PRL-independent Nb2-Sp cell line. The molecular basis of rapamycin resistance in Nb2-Sp cells is not known. However, resistance to growth inhibition by rapamycin may arise from mutations in TOR/mTOR (Luo *et al.* 1996) or any of its downstream components including Tap42, FKBP and p70S6K (Heitman *et al.* 1991, Dumont *et al.* 1995, Fruman *et al.* 1995, Di Como & Arndt 1996, Brennan *et al.* 1999). Introduction of the murine α4 cDNA into Jurkat cells or the increased association of PP2Ac/α4 in culture with low serum concentration have also been reported to confer rapamycin resistance to cells (Inui *et al.* 1998). The rapamycin sensitivity of the α4–PP2A interaction is controversial. While formation of a GST-α4 fusion protein with PP2A in COS-7 or Jurkat cell lines was shown to be rapamycin sensitive (Murata *et al.* 1997, Inui *et al.* 1998), α4–PP2A interaction in human embryonic kidney 293 cells was shown to be constitutive and rapamycin insensitive (Nanahoshi *et al.* 1998). Recently, mTOR was shown to be a direct target of the phosphoinositide 3′-kinase

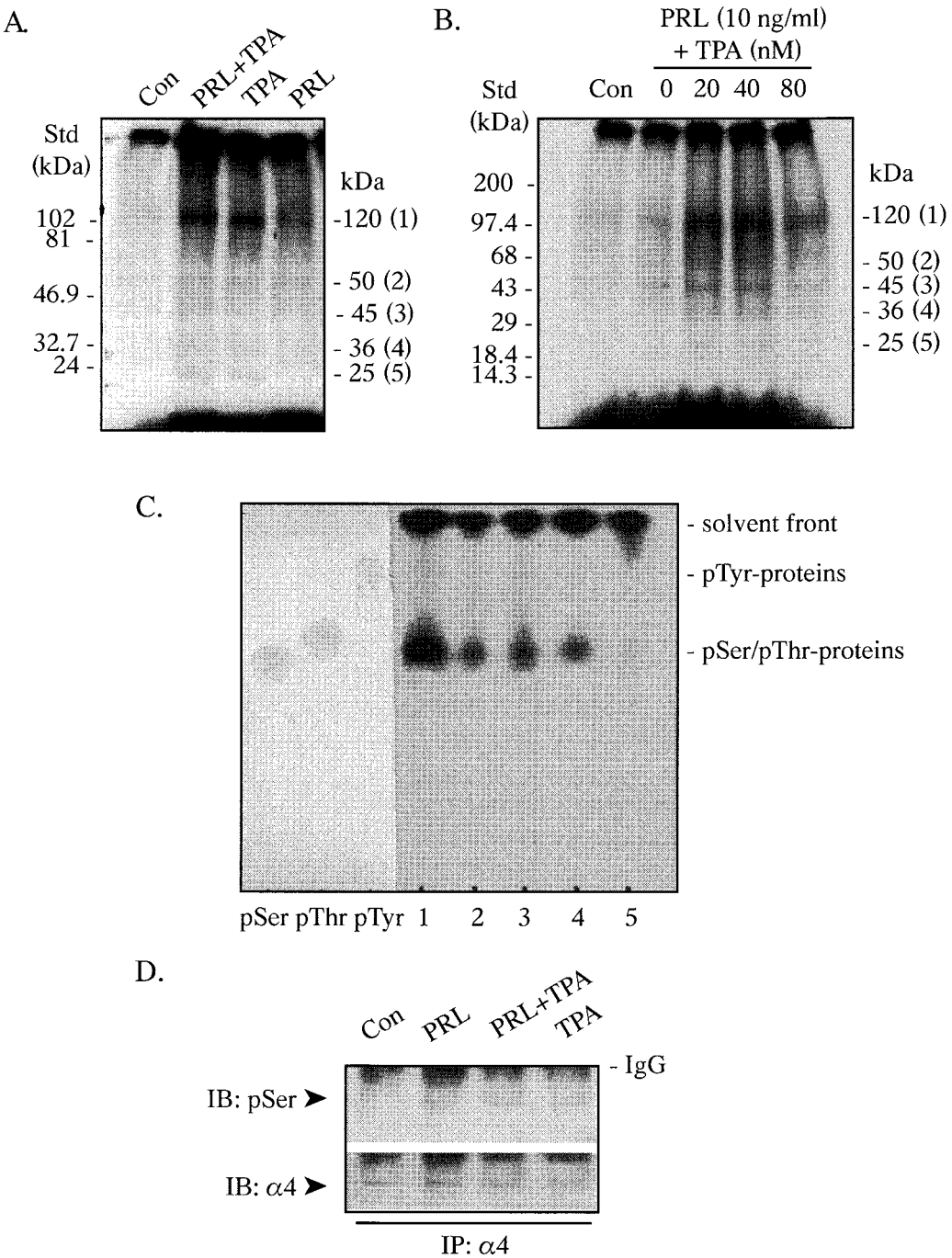

Figure 8 PRL-/TPA-inducible phosphorylation of α4. (A, B) Nb2 cells were radiolabelled with [^{32}P]-orthophosphate in the presence of PRL (10 ng/ml) and/or TPA (A, 20 nM; B, as indicated) for 20 min. Controls (Con) were untreated. Total cell lysates were immunoprecipitated with anti-α4 antibodies. Immuno-complexes were analyzed by SDS-PAGE and autoradiography. The MWs of the radiolabelled bands (1–5) are indicated on the right. (C) Cells were radiolabelled in the presence of PRL+TPA (40 nM). Anti-α-immunoprecipitated bands 1–5 from triplicate samples were excised from the gel for phosphoamino acid analysis. Thin layer chromatography and autoradiography of acid-hydrolyzed samples and standards (pSer, pThr and pTyr) were performed. (D) Immunopreciptation (IP) and blotting (IB) of Nb2 cell lysates after PRL ± TPA (20 nM) treatment. Each set of data is representative of two to three independent experiments.

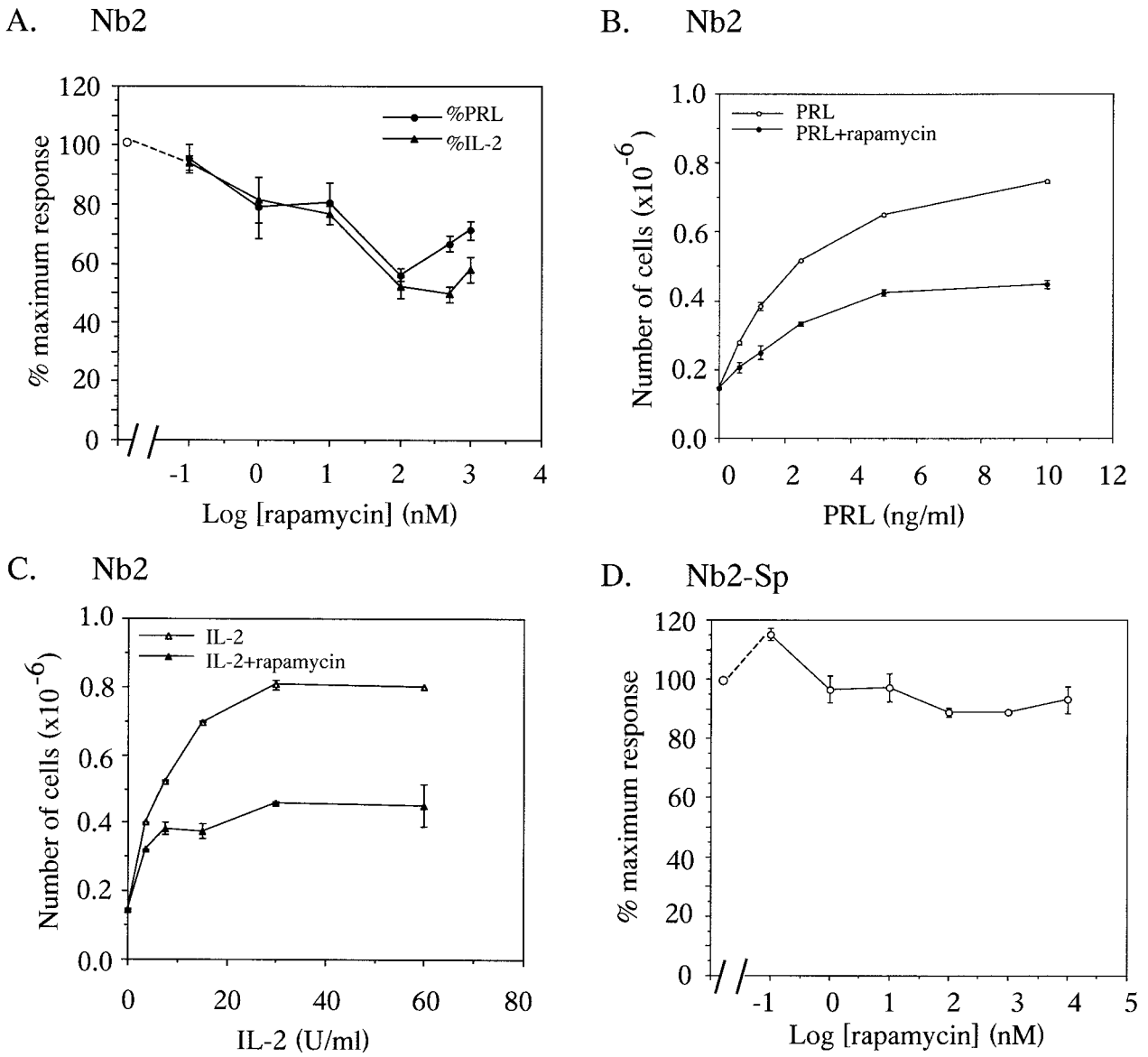

Figure 9 Rapamycin inhibits proliferation of Nb2 but not Nb2-Sp cells. (A) Quiescent Nb2 cells were treated with PRL (10 ng/ml) or IL-2 (30 U/ml) and increasing concentrations of rapamycin. Controls received mitogen alone. Cell count was determined in a 3-day bioassay and data plotted as a % of the maximum response seen in controls. (B and C) Quiescent Nb cells were treated with 100 nM rapamycin with increasing doses of (B) PRL or (C) IL-2 for a 3-day bioassay. Controls received no rapamycin. (D) Nb2-Sp cells were treated with rapamycin while controls were untreated. Data are plotted as in (A). Each graph is representative of two to three independent experiments, each treatment done in triplicate. Values are means ± S.D.

(PI3K)–AKT/protein kinase B signalling pathway in mitogen-stimulated cells and the activation status of the PI3K–AKT pathway in cancer cells was suggested as an important determinant of cellular sensitivity to rapamycin (Sekulic *et al.* 2000).

Our study also demonstrated that overexpression of the α4 protein in COS-1 cells inhibited PRLr signalling to the IRF-1 promoter, an effect that was not due to global protein shutdown since the transfected Stat1α protein remained elevated. Transient overexpression of α4 in COS-7 cells has also been reported to have no effect on protein synthesis (Chung *et al.* 1999). Taken together, PRL downregulation of α4 mRNA and protein and/or the modulation of α4 protein action through PRL-inducible phosphorylation may facilitate activation of PRL-responsive genes (e.g. IRF-1) culminating in Nb2 cell proliferation. The constitutive expression of α4 in Nb2-Sp cells may not be inconsistent with this model, for

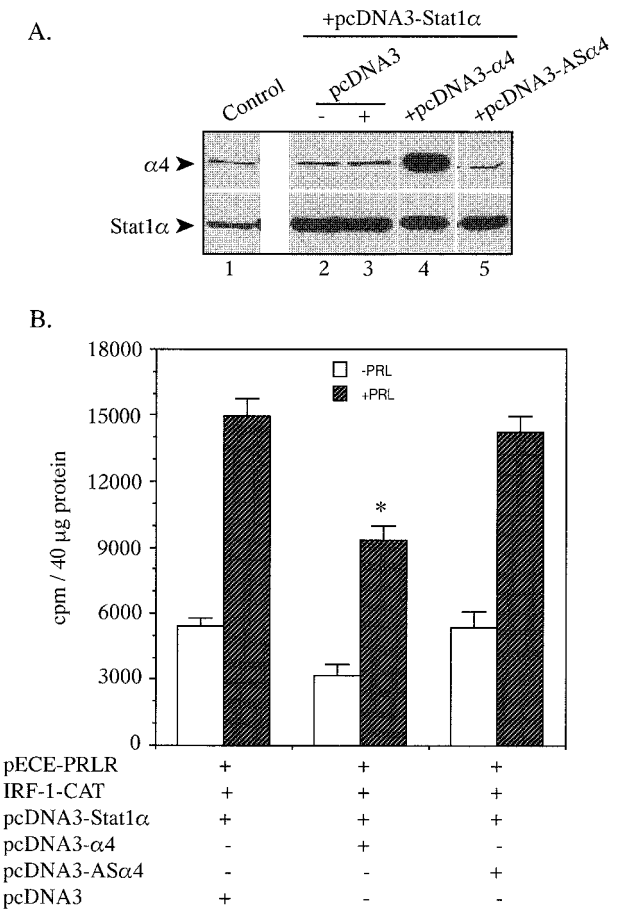

Figure 10 Overexpression of α4 inhibits IRF-1 promoter activity. COS-1 cells were transiently transfected with expression contructs of the Nb2 form of the PRLr, 1·7 kb IRF-1–CAT reporter, Stat1α and either α4 or antisense α4 or the pcDNA3 vector. Untransfected COS-1 cells served as controls. Cells were made quiescent overnight in medium containing 1% horse serum prior to treatment with or without PRL (100 ng/ml) for 24 h. Cell lysates were prepared for (A) Western analysis or (B) CAT enzyme activity. In (A), the immunoblot (20 μg protein/sample; − PRL) was probed sequentially with anti-α4 or anti-Stat1α antibodies. Lane 1, untransfected COS-1 cells; lanes 2–5, COS-1 cells transfected with pcDNA3-Stat1α and with (+) or without (−) additional pcDNA3 vector (lanes 2 and 3) or with pcDNA3-α4 (lane 4) or antisense pcDNA3-ASα4 (lane 5). In (B), CAT assay was performed with equal amounts of protein for each sample. Representative of four independent experiments, with each treatment assayed in triplicate. Values are means ± S.D. *$P<0·05$.

these cells are insensitive to rapamycin, indicating that mTOR signalling may be defective or sub-optimal in Nb2-Sp cells. Inhibition of PP2A, the α4 partner, has been shown to induce serine/threonine phosphorylation, subcellular redistribution and functional inhibition of Stat3 (Woetmann *et al.* 1999). The role of the mTOR components (α4/PP2A) in modulating PRLr action through Stat activity is under further investigation.

Acknowledgements

We would like to thank Dr Li-yuan Yu-Lee (Baylor College of Medicine) for excellent advice and for kindly providing the constructs and COS-1 cells used for the CAT assays. R T M B was supported in part by a Cancer Research and Education (CaRE)–Nova Scotia trainee award, with funding from the Faculty of Medicine, Dalhousie University. L M J was supported by a CaRE–Nova Scotia Summer Studentship. C K L T was a Scholar of the Canadian Institutes of Health Research (CIHR). This study was supported by CIHR grant MOP12895 (CKLT).

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Received 26 November 2001
Accepted 5 February 2002