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PII: S0303-7207(09)00307-4
DOI: doi:10.1016/j.mce.2009.05.021
Reference: MCE 7235

To appear in: *Molecular and Cellular Endocrinology*

Received date: 16-12-2008
Revised date: 28-5-2009
Accepted date: 29-5-2009

Please cite this article as: Maroni, P., Citterio, L., Piccoletti, R., Bendinelli, P., SAM68 AND ERKs REGULATE LEPTIN-INDUCED EXPRESSION OF OB-Rb mRNA IN C2C12 MYOTUBES, *Molecular and Cellular Endocrinology* (2008), doi:10.1016/j.mce.2009.05.021

# SAM68 AND ERKs REGULATE LEPTIN-INDUCED EXPRESSION OF OB-Rb mRNA IN C2C12 MYOTUBES

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**Abstract**

Acute leptin treatment significantly increases the mRNA of the long isoform of leptin receptor (OB-Rb) in C2C12 myotubes after as little as 30 minutes, without affecting that of the short isoform (OB-Ra). The Sam68 STAR protein has been implicated in leptin signal transduction as an adaptor molecule useful to recruit other signalling proteins. We found that leptin increased Sam68 tyrosine-phosphorylation so decreasing its poly(U)-binding capacity. RT-PCR analysis of the mRNA bound to immunoprecipitated Sam68 showed that Sam68 associated with OB-Rb but not OB-Ra mRNA in control and leptin-treated C2C12 cells. The siRNA-mediated silencing of Sam68 reduced its levels by 89% and abolished the leptin-mediated increase in OB-Rb mRNA. Leptin activates ERKs which in turn might phosphorylate Sam68 modifying its influence on mRNA. We did not observe any changes in Sam68 Ser/Thr phosphorylation but using the specific MEK1 inhibitor PD-98059 showed that leptin-mediated ERK activation is essential for leptin's effect on OB-Rb mRNA expression. Thus it appears that leptin has a positive short-term effect on the regulation of OB-Rb mRNA in C2C12 cells, involving both Sam68 and ERKs. These results might suggest that leptin signal acutely favours its own sensitivity.

*Keywords*: leptin; leptin receptor; Sam68; ERKs.

## 1. Introduction

Leptin receptor (OB-R), the product of the *db* gene (Lee et al., 1996), is a member of the class I cytokine receptor family (Tartaglia, 1997). As a result of the alternative splicing of the RNA transcript of *db*, OB-R has at least six isoforms, which have the same extracellular ligand-binding domain but distinct cytoplasmatic region domains. The long isoform OB-Rb has 306 amino acids at its carboxyl terminal, whereas the short forms have 32-40 amino acid cytosolic residues (Friedman, 1998; Hegyi et al., 2004). Despite the widespread tissue distribution and abundance of the OB-Ra short form, little is known about its physiological significance whereas OB-Rb is highly expressed in the hypothalamus (Bjørbæk and Kahn, 2004) and seems to be the only one capable of fully signalling (Hegyi et al., 2004). Leptin receptors are most abundantly expressed in the brain, but are also present in a number of peripheral tissues where they directly mediate a wide range of leptin roles (Margetic et al., 2002).

The processes of gene expression, from mRNA biogenesis to translation and degradation, revolve around messenger ribonucleoprotein complexes. RNA-binding proteins transport coding and non-coding RNAs to their cellular destinations (Liu et al., 2006; Cao et al., 2006), regulate mRNA stability (Goldstrohm et al., 2006), and control translation in response to environmental cues (Antar et al., 2004). It is also known that most RNA-binding proteins are components of multi-protein complexes which include other RNA-binding proteins and a plethora of RNA targets.

It has recently been reported that leptin transduction signalling involves Sam68 (Src-associated in mitosis) as the docking protein for PI3K activation in mononuclear cells (Sánchez-Margalet et al., 2003), and that Sam68 is recruited by insulin (Sánchez-Margalet and Najib, 2001) and angiotesin II receptors (El Mabrouk, et al., 2004). Sam68 also has nuclear functions as it is one of the members of the STAR (signal transducers and activators of RNA metabolism) family of RNA-binding proteins which regulate aspects of RNA metabolism including pre-mRNA splicing and polyadenylation, RNA transport and stability, and RNA translation (Lukong and Richard, 2003; Paronetto et al., 2006; Paronetto et al., 2007).

The aim of this study was to investigate whether leptin affects Sam68 also as an RNA modulating protein and, in particular, whether Sam68 may affect the regulation of alternatively spliced isoforms of leptin receptors. To this end, we studied the effects of acute leptin administration on the mRNA expression of OB-Ra and OB-Rb and its relationship with

Sam68 in C2C12 cells, a mouse skeletal muscle cell line that is responsive to the hormone (Berti and Gammeltoft, 1999; Maroni et al., 2005; Bendinelli et al., 2005).

## 2. Materials and methods

### *2.1. Materials*

The recombinant human leptin came from Pharma Biotechnologie Hannover (Hannover, Germany); the anti-Sam68 (C-20), anti-ERK (K-23), and anti-phosphoERK (Tyr204) antibodies from Santa Cruz Biotechnology (Santa Cruz, CA); the anti-phosphotyrosine (RC20) antibodies cross-linked to horseradish peroxidase from Transduction Laboratories (Lexington, KY); the anti-phosphoSer/Thr-Pro MPM-2 antibodies from Upstate Biotechnology Inc. (Lake Placid, NY); and the PD-98059 from Biomol (Plymouth Meeting, PA). The anti-tropomyosin antibodies (36/39 kDa), agarose-immobilised poly(U), and actinomycin D from Sigma Chem. Co. (St. Louis, MO).

### *2.2. Cell culture and treatment*

The mouse C2C12 myoblasts (ATCC, Rockville, MD) were grown and differentiated as described (Maroni et al., 2005; Bendinelli et al., 2005). C2C12 myotubes were treated with PBS (control) or leptin (50 ng/ml in PBS) for 30 minutes. To inhibit ERKs, 10 μM of PD-98059 were added to the cells 30 min before leptin treatment. To evaluate the Ob-Rb steady state, 5μg/ml actinomycin D was added 30 min before leptin treatment (Cheadle et al., 2005).

### *2.3. RNA extraction and semiquantitative RT-PCR analysis*

Total RNA was extracted using an RNeasy kit (Qiagen, Germany) and estimated by measuring adsorbance at 260 nm. The RNA purity was checked by the ratio between adsorbance at 260 and 280 nm (normal range 1.8-2.1), and its integrity by 1% agarose gel. Semi-quantitative RT-PCR was used to analyse the expression of isoform-specific OB-Rb and OB-Ra mRNA. Reverse transcription was carried out at 25°C for 10 min, 48°C for 30 min and 95°C for 5 min, using 2 μg of total RNA and the TaqMan® reverse transcription kit (Applied Biosystems, Foster City, CA); 4 μl of each reverse transcription reaction product

was PCR amplified using primers chosen on the basis of the published sequence of mouse OB-Rb (GenBank accession no. **U58861**) and OB-Ra (GenBank accession no. **U58862**) (Berti and Gammeltoft, 1999). Oligonucleotides corresponding to the divergent COOH terminus were used for the OB-Rb and OB-Ra cDNAs (sense primer 5'-TTTTTACCAAGCACGCAGAATC-3' and antisense primer 5'-ACCCCGAGAATGAAAGTTGTG-3', which generated a product of 477 bp for OB-Rb; and sense primer 5'-TGGCCCATGAGTAAAGT-3' and antisense primer 5'-CTGTTGGGAAGTTGGTAG-3', which generated a product of 545bp for OB-Ra) (Berti and Gammeltoft, 1999). For each sample, cyclophilin mRNA was amplified as an internal control (sense primer 5'-ACCCCACCGTGTTCTTCGAC-3' and antisense primer 5'-CATTTGCCATGGACAAGATG-3) (Lee et al., 2005). The PCRs were carried out on a GeneAmp PCR System 9600 (Applied Biosystems-Foster City-CA) using 1U of *Taq* DNA Polymerase (Bioline; UK) at the following temperatures: 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, with an additional 7 min extension step at 72°C. The number of cycles was optimised to ensure amplification in the exponential phase of PCR: 35 cycles for Ob-Ra and Ob-Rb, and 23 cycles for cyclophilin. A sample was run omitting cDNA to detect any contamination in the reagents. The same sample was amplified in triplicate and assayed several times. The amplified fragments were separated by electrophoresis on 2% agarose gel containing ethidium bromide and visualised using a digital imaging system (Labworks™-UVP Bioimaging Systems, Upland, CA). Densitometric analysis was performed as described below and the signal intensity of each sample was normalised to the relative cyclophilin signal.

*2.4. Poly(U) binding assay*

The cells were lysed in 1% Triton X-100, 100 mM NaCl, 10 mM $MgCl_2$, 30 mM Tris, pH 7.5, 1 mM DTT, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM $Na_3VO_4$, and 100 mM NaF. The lysates were pre-cleared using protein A-Sepharose, and then 20 μl of a 50% solution of agarose-poly(U) beads were added to 100 μg of proteins of cellular lysates. The mixture was rotated for 1 hour at 4°C. After several washing the beads were analysed by immunoblot for associated Sam68.

*2.5. Immunoblot analysis*

The immunoprecipitates, poly(U) agarose beads and cell lysates were used for immunoblot analysis (Bendinelli et al., 2005). After detection with anti phospho-Tyr, the same blot was re-probed with anti-Sam68 antibodies (Bendinelli et al., 2005)

*2.6. Immunoprecipitation of mRNA-Sam68 complexes*

This was performed as described by Paronetto et al. (2006). Cellular extract (7 mg of proteins obtained with 0.5 % Triton X-100, 10 mM $MgCl_2$, 150 mM NaCl, 5 mM EGTA, 1 mM DTT, 1 mM $Na_3VO_4$, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 100mM NaF, and 100U/ml RNasin) were cleared with protein A-Sepharose (in the presence of 1 μg/ml tRNA) and then with 15 μg of unspecific IgG preadsorbed to protein A-Sepharose. Samples were incubated with 15 μg of anti-Sam68 antibodies preadsorbed to protein A-Sepharose beads or 15 μg of unspecific IgG preadsorbed to protein A-Sepharose (Mock precipitation). After several washing, the samples were incubated with 50 μg of protease K, after which the RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) and used for PCR analysis as described above.

*2.7. siRNA transient transfection*

Before transfection, the C2C12 myotubes were trypsinised and diluted in six-well plates to give 30-50% confluence 24 hours after plating. Stealth Sam68 small interfering RNA (Khdrbs1 Stealth Select RNAi, Invitrogen, Carlsbad, CA) was transfected at a concentration of 30 nM with the Lipofectamine RNAiMax. The control cells were transfected with a control siRNA duplex consisting of identical nucleotides in a scrambled order with no known target. After 48 hours cells were processed for immunoblot analysis and for RNA extraction followed by PCR analysis.

*2.8. Other procedures*

The immunoreactive bands and the bands visualised by means of ethidium bromide staining were quantitatively measured using densitometry and Image Master Software (GE-Healthcare, Bucks, UK); the volumetric measurements (OD x $mm^2$) are expressed as the

leptin-induced fold increase over control values. The absolute values obtained in control and treated cells varied in the range of 10-15% in the different experiments. The data from all of the experiments (mean values ± SEM of at least five separate experiments) were compared using one-way analysis of variance (ANOVA) followed by Bonferroni's *t*-test. P values of <0.05 were considered statistically significant.

## 3. Results

### *3.1. Effect of leptin treatment on mRNA expression for OB-Ra and OB-Rb*

The expression of mRNA for the leptin receptors OB-Ra and OB-Rb in C2C12 cells was analysed by means of semi-quantitative RT-PCR, which is highly sensitive in detecting alternatively spliced isoforms. The PCR assays were optimised for semi-quantitative analysis by testing different numbers of PCR cycles for each mRNA (data not shown), and mRNA expression was standardised using cyclophilin as an internal control for reaction efficiency. C2C12 cells express mRNA for both the long and short leptin receptor isoforms (Fig. 1A), but 30 minutes of leptin treatment induced a three-fold increase in the amount of mRNA for the long isoform without affecting the level of mRNA for the short isoform (Fig. 1B). To analyse the possible influence of the Ob-Rb steady state modification on leptin effect, we examined the response to actinomycin D. The pre-treatment of C2C12 cells with actinomycin D 30 min before leptin administration, completely inhibited the increase of mRNA expression for Ob-Rb hormone-induced (Fig. 1C), suggesting an insignificant contribute of the altered mRNA stability to increase of Ob-Rb mRNA expression.

### *3.2. Effect of leptin treatment on Sam68 tyrosine-phosphorylation and poly(U) binding*

Total extracts from control and leptin-treated C2C12 cells were immunoprecipitated with anti-Sam68 antibodies, and analysed by means of immunoblotting with anti-phosphotyrosine antibodies. Leptin treatment doubled the tyrosine-phosphorylation of Sam68 (Fig. 2A), thus indicating that the hormone signalling involves the protein in skeletal muscle cells. Tyrosine-phosphorylation of Sam68 negatively regulates the association between the protein and mRNA (Wang et al.,1995), and so we analysed the capacity of Sam68 to bind poly(U) after leptin treatment as it is known that Sam68 specifically binds this RNA polymer (Taylor and Shalloway, 1994). The cell lysates were incubated with poly(U)-agarose beads and analysed

by means of immunoblotting with anti-Sam68 antibodies. Poly(U)-agarose beads efficiently bound Sam68 in control cell lysates, but leptin treatment significantly reduced the binding of Sam68 to poly(U) by 30% (Fig. 2B).

*3.3. Studies of the possible role of Sam68 in leptin-induced regulation of OB-Rb mRNA*

In order to demonstrate a link between Sam68 and OB-Rb mRNA, we immunoprecipitated Sam68 from C2C12 cell lysates prepared under conditions that preserved the integrity of RNA, which was then extracted from the immunoprecipitates and underwent RT-PCR in order to detect the presence of OB-Ra and OB-Rb mRNA. Mock immunoprecipitations with unspecific IgGs did not reveal any mRNA (Fig. 3A), but OB-Rb mRNA was detected in the Sam68 immunoprecipitates obtained from control and leptin-treated cells even though no differences were visualised between the control and leptin-treated samples; OB-Ra mRNA was never detected.

We therefore investigated the effect of reducing Sam68 levels by means of Sam68 siRNA transfection on the leptin-induced increase in OB-Rb mRNA. C2C12 cells transiently transfected with Sam68-specific siRNA showed an 89% reduction in the level of the protein as shown in Figure 3B, and the fact that non-specific siRNA had no effect (not shown) indicated the success of the RNAi knock-down. After siRNA transfection, the cells were treated with leptin and the extracted RNA was analysed by means of PCR with primers for OB-Rb. The Sam68 knock-down impaired the leptin-induced increase in OB-Rb mRNA (Fig. 3C), thus suggesting that leptin involves Sam68 in regulating the mRNA for its own receptor.

*3.4. Effect of ERKs on the leptin-induced increase in OB-Rb mRNA*

We have previously shown that leptin activates ERKs in C2C12 cells (Maroni et al., 2005) and, as the Sam68-phosphorylation by ERKs may modulate the Sam68 RNA-binding capacity and regulate mRNA splicing (Matter et al., 2002; Shin and Manley, 2004), we studied the Ser/Thr-phosphorylation of Sam68 after leptin treatment. Total cell extracts were immunoprecipitated with anti-Sam68 antibodies and then analysed by means of immunoblotting with an antibody that recognises phosphoserine/phosphothreonine followed by proline. Leptin tended to increase the Ser/Thr phosphorylation of Sam68, but the variability of the response prevented any clear findings (data not shown). Nevertheless, in order to detect a role for ERKs in regulating the leptin-induced increase in OB-Rb mRNA, we

evaluated mRNA expression after ERK inhibition. We treated C2C12 cells with PD-98059, an effective and specific MEK1 inhibitor (Dudley et al., 1995) before leptin treatment and found that the presence of the ERK inhibitor prevents the leptin-induced increase both in ERK phosphorylation and in OB-Rb mRNA (Fig. 4B).

## 4. Discussion

We first confirmed that both OB-Ra and OB-Rb mRNAs are expressed in C2C12 cells under baseline conditions, in line with the findings of others in peripheral tissues, including skeletal muscle (Berti and Gammeltoft, 1999; Frühbeck et al., 1999; Ceddia 2005), and then showed that acute leptin administration rapidly triples the expression of OB-Rb mRNA.

There are various published data concerning the ability of leptin to control its own receptor isoforms, most of which indicate the induction of a common homologous mechanism of down-regulation which, together with others, may explain the leptin-resistance observed in obesity. For example, it has been reported that the levels of OB-Rb mRNA are negatively regulated by leptin in neuroblastoma cells (Hikita et al., 2000) within 12 hours, and that both leptin receptor mRNA and protein are down-regulated in the hypothalamus of rats receiving long-term leptin treatment (Martin et al., 2000). Leptin down-regulates the expression of both OB-Rb and OB-Ra mRNA within 24 hours in HepG2 cells (Liu et al., 2004). The levels of the overall OB-R mRNA and specific OB-Rb mRNA decreased in testicular tissue taken from 30-day-old rats and incubated for 120 minutes in the presence of recombinant leptin (Tena-Sempere et al., 2001).

Differently from the above findings, our data show that leptin significantly increases the OB-Rb mRNA expression as early as after 30 minutes. This effect may be attributable to the times of observation: it is possible to hypothesise that leptin early triggers some mechanisms that favor cell sensitivity to the hormone as the long receptor isoform is more competent in signalling transduction than the short receptor.

One possible candidate for integrating signal transduction pathways and RNA metabolism is Sam68, a member of the STAR protein family that not only contains KH domains, but also proline- and tyrosine-rich sequences that respectively interact with RNA and proteins (Lukong and Richard, 2003). Sam68 may therefore play a role in RNA metabolism including alternative splicing (Gorla et al., 2006; Rajan et al., 2008), as well as in signal transduction as an adaptor protein (Sánchez-Margalet and Najib, 2001).

It has been reported that leptin stimulates the Tyr-phosphorylation of Sam68 and that this is followed by the association of the protein with STAT-3, IRS-1 and PI3K, as well as by a decrease in the capacity of Sam68 RNA to bind to poly(U) in peripheral blood mononuclear cells (Martin-Romero and Sánchez-Margalet, 2002). In line with this, we found that the phosphotyrosine content of Sam68 in C2C12 cells increases 30 minutes after leptin treatment at the same time as its poly(U) binding activity decreases.

To detect a possible direct correlation between Sam68 and the variation in leptin receptor mRNA levels after leptin treatment, we investigated the association of Sam68 with OB-R mRNA. We found that Sam68 associated with OB-Rb mRNA in both control and leptin-treated C2C12 cells, whereas no association was found with OB-Ra mRNA before or after leptin treatment. The leptin-induced tyrosine-phosphorylation of Sam68 does not negatively influence the binding of the protein to OB-Rb mRNA. Nevertheless, we found that Sam68 does play a role in the leptin-induced regulation of OB-Rb mRNA in C2C12 cells as we were able to show that its 90% knock-down by means of siRNA-mediated gene silencing reduced the leptin-induced increase in OB-Rb mRNA levels, a finding indicating a previously unknown leptin-induced role for Sam68.

Sam68 has also been identified as a novel ERK target: Sam68 phosphorylation by ERKs regulates the alternative splicing of exon v5 in CD44 (Matter et al., 2002; Shin and Manley, 2004) without changing the protein's RNA-binding activity (Matter et al., 2002). Furthermore, Sam68 is phosphorylated by ERKs in spermatocytes and co-sediments with polysomes during meiotic division (Paronetto et al., 2006).

We have previously shown that acute leptin administration rapidly activates ERKs in mouse skeletal muscle and C2C12 cells (Maroni et al., 2003; Maroni et al., 2005), and so we looked for a possible relationship between ERK activation and Sam68 by analysing the Ser/Thr-phosphorylation status of Sam68 in C2C12 cells after 30 minutes of leptin treatment. No statistically significant change in Ser/Thr phosphorylation was identified. However, we did find that ERK activation is essential for the effect of leptin on OB-Rb mRNA expression as the administration of PD-98059, a specific MEK1 inhibitor, before leptin treatment prevented the increase both in the ERK phosphorylation and in the levels of receptor mRNA induced by the hormone.

We suggest three ways in which the discrepancy in these results can be interpreted: i) Sam68 Ser/Thr phosphorylation by ERKs is a transient phenomenon and thus hardly detectable (Tiesserant and König, 2008); (ii) only a small portion of Sam68 can be obtained in its phosphorylated form (Tiesserant and König, 2008); and iii) the relationship between ERKs

and Sam68 may be indirect and so ERKs may be influencing the processing of mRNA by Sam68 through the phosphorylation of an intermediate factor.

In conclusion, our findings suggest that the positive regulation of OB-Rb mRNA by leptin is one of the early events triggered by the hormone in C2C12 muscle cells, and depends on both Sam68 and ERKs. We are now investigating the relationship between Sam68 and ERKs.

**Acknowledgements**

This work was supported by Grants from Ministero Istruzione, Università e Ricerca.

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**Figure Legends**

Fig. 1. OB-Ra and OB-Rb mRNA expression in C2C12 cells control (A) and leptin-treated for 30 minutes (B). Effect of actinomycin D pre-treatment on Ob-Rb mRNA expression (C). Total RNA was reverse transcribed and amplified with OB-Ra and OB-Rb specific primers: the expression of cyclophilin was used as a reference control. The histogram show the densitometric analysis of the PCR products (OD x $mm^2$) expressed as the mean percentages ± SEM (normalised for cyclophilin) of the control value of three separate experiments. * $p<0.05$ *vs* controls.

C: control cells; L30': leptin-treated cells for 30 minutes; St: 1 kb ladder.

Fig. 2. Effect of leptin on the tyrosine-phosphorylation (A) and poly(U)-binding (B) of Sam68 in C2C12 cells. A) Cell extract proteins immunoprecipitated with anti-Sam68 antibodies (αSam68) were analysed by means of immunoblotting with αSam68 and anti-phosphotyrosine (αpTyr) antibodies. B) Cell extract proteins were incubated with poly(U)-agarose beads and analysed by means of immunoblotting with anti-Sam68 antibodies. The histograms show the densitometric analysis of the immunoreactive bands (OD x $mm^2$) expressed as the mean percentages ± SEM of the control value of three separate experiments. * $p<0.05$ *vs* controls.

C: control cells; L30': leptin-treated cells for 30 minutes; St: 1 kb ladder.

Fig. 3. Sam68 involvement in leptin-induced regulation of OB-Rb mRNA. (A) Expression of OB-Ra and OB-Rb mRNA co-precipitated with unspecific IgG (Mock) and anti-Sam68 antibodies (αSam68). The RNAs extracted from the imunoprecipitates obtained using unspecific IgG and anti-Sam68 antibodies were reverse transcribed and amplified with OB-

Ra- and OB-Rb-specific primers. (B) siRNA knock down of Sam68 visualised by means of immunoblotting. Tropomyosin was used to verify equal protein loading. (C) OB-Ra and OB-Rb mRNA expression after siRNA knock down of Sam68.

C: control cells; L30': leptin-treated cells for 30 minutes; St: 1 kb ladder.

Fig. 4. Effect of ERK inhibition on the expression of OB-Rb mRNA in control and leptin-treated cells. The cells were untreated and treated with 10 μM PD-98059 30 minutes before leptin treatment. (A) Cell extract proteins were analysed by means of immunoblotting with anti-ERKs and anti-phosphoERKs antibodies. (B) Total RNA was reverse transcribed and amplified with OB-Rb specific primers: the expression of cyclophilin was used as a reference control. The histogram show the densitometric analysis of the PCR products (OD x $mm^2$) expressed as the mean percentages ± SEM (normalised for cyclophilin) of the control value of three separate experiments. * $p<0.05$ *vs* controls.

C: control cells; L30': leptin-treated cells for 30 minutes; St: 1 kb ladder.

**Figure 1**

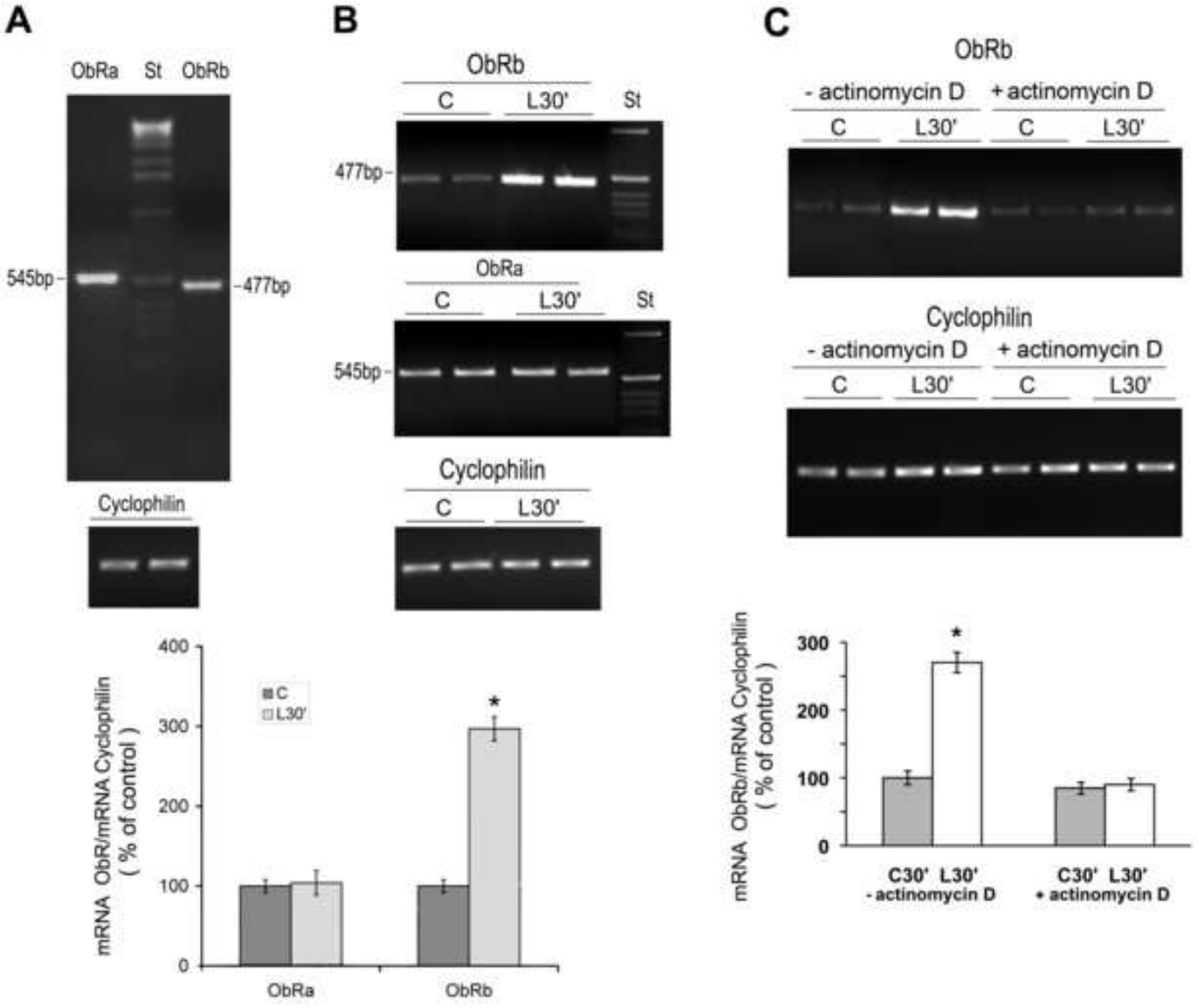
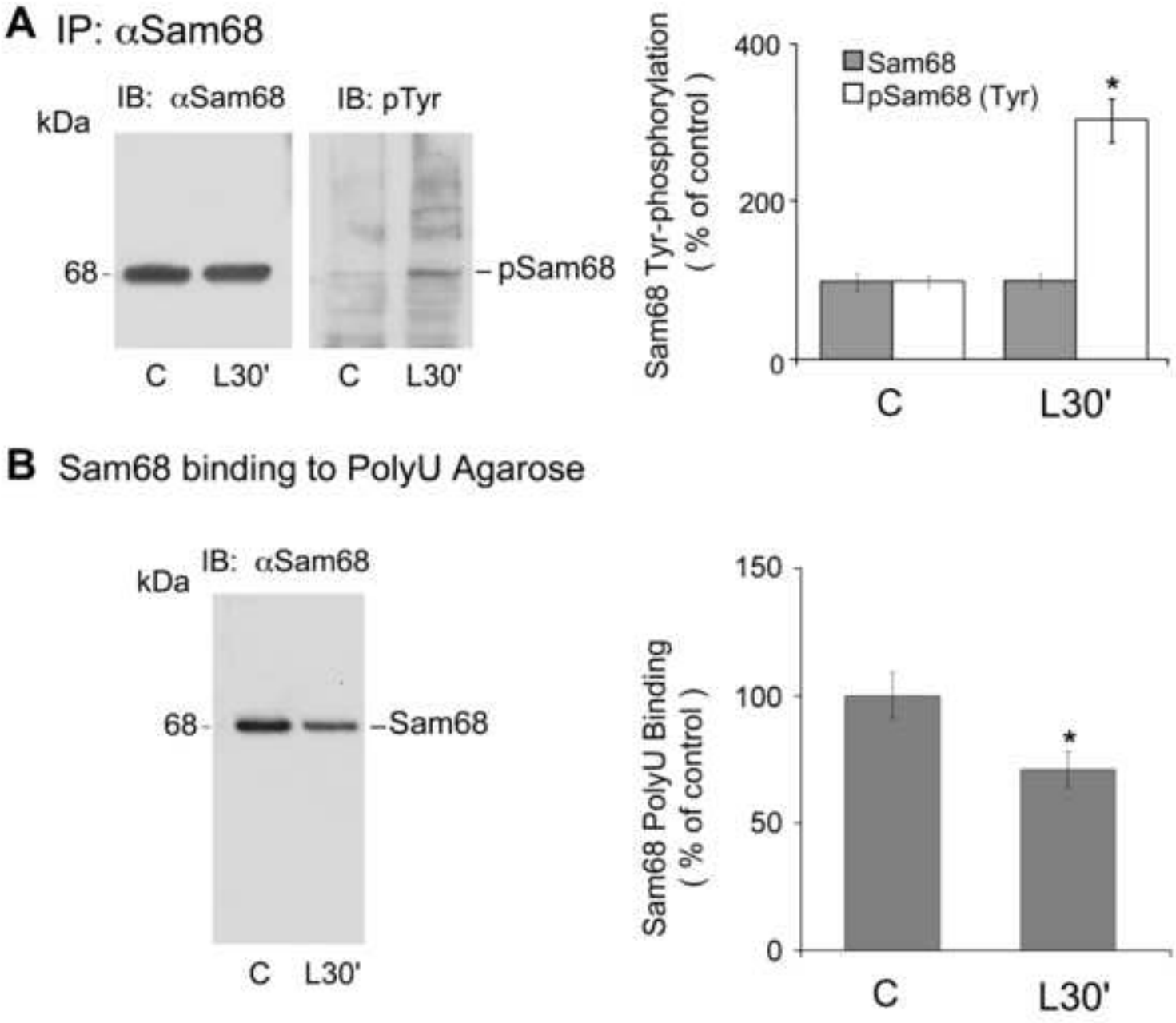

A
ObRa
St
ObRb
545bp
477bp
Cyclophilin
B
ObRb
C
L30'
St
477bp
ObRa
C
L30'
St
545bp
Cyclophilin
C
L30'
mRNA ObR/mRNA Cyclophilin ( % of control )
400
300
200
100
0
C
L30'
*
ObRa
ObRb
C
ObRb
- actinomycin D
+ actinomycin D
C
L30'
C
L30'
Cyclophilin
- actinomycin D
+ actinomycin D
C
L30'
C
L30'
mRNA ObRb/mRNA Cyclophilin ( % of control )
300
200
100
0
*
C30' L30'
- actinomycin D
C30' L30'
+ actinomycin D

Figure 2


A IP: αSam68
kDa
IB: αSam68
IB: pTyr
68
pSam68
C
L30'
C
L30'
Sam68 Tyr-phosphorylation ( % of control )
400
200
0
Sam68
pSam68 (Tyr)
*
C
L30'
B Sam68 binding to PolyU Agarose
kDa
IB: αSam68
68
Sam68
C
L30'
Sam68 PolyU Binding ( % of control )
150
100
50
0
*
C
L30'