Translational control of the sterol-regulatory transcription factor SREBP-1 mRNA in response to serum starvation or ER stress is mediated by an internal ribosome entry site

SREBPs (sterol-regulatory-element-binding proteins) are a family of transcription factors that modulate the expression of several enzymes implicated in endogenous cholesterol, fatty acid, triacylglycerol and phospholipid synthesis. In the present study, evidence for SREBP-1 regulation at the translational level is reported. Using several experimental approaches, we have demonstrated that the 5′-UTR (untranslated region) of the SREBP-1a mRNA contains an IRES (internal ribosome entry site). Transfection experiments with the SREBP-1a 5′-UTR inserted in a dicistronic reporter vector showed a remarkable increase in the downstream cistron translation, through a cap-independent mechanism. Insertion of the SREBP-1c 5′-UTR in the same vector also stimulated the translation of the downstream cistron, but the observed effect can be ascribed, at least in part, to a cryptic promoter activity. Cellular stress conditions, such as serum starvation, caused an increase in the level of SREBP-1 precursor and mature form in both Hep G2 and HeLa cells, despite the overall reduction in protein synthesis, whereas mRNA levels for SREBP-1 were unaffected by serum starvation. Transfection experiments carried out with a dicistronic construct demonstrated that the cap-dependent translation was affected more than IRES-mediated translation by serum starvation. The thapsigargin- and tunicamycin-induced UPR (unfolded protein response) also increased SREBP-1 expression in Hep G2 cells, through the cap-independent translation mediated by IRES. Overall, these findings indicate that the presence of IRES in the SREBP-1a 5′-UTR allows translation to be maintained under conditions that are inhibitory to cap-dependent translation.

Key words: gene expression, internal ribosome entry site (IRES), sterol-regulatory-element-binding protein-1 (SREBP-1), translational regulation, unfolded protein response (UPR), 5′-untranslated region (5′-UTR).

INTRODUCTION

Lipid homeostasis in vertebrate cells is regulated by a family of membrane-bound transcription factors designated as SREBPs (sterol-regulatory-element-binding proteins). SREBPs directly activate the expression of more than 30 genes implicated in the synthesis and uptake of cholesterol, fatty acids, triacylglycerols (triglycerides) and phospholipids [1–3]. The SREBP family of bHLH-LZ (basic helix–loop–helix-leucine zipper) transcription factors consists of SREBP-1a, SREBP-1c and SREBP-2, encoded in the mammalian genome by two genes, Srebf1 and Srebf2. SREBPs differ in their tissue-specific expression, target-gene selectivity and the relative potency of their transactivation domains [4–6]. SREBP-1a is constitutively expressed at low levels in liver and in most tissues of adult animals, and is the predominant isoform in most cultured cell lines. SREBP-1c expression is finely and strictly regulated in experimental animals and humans in response to diet and hormones [4–9]. SREBPs are synthesized as inactive precursors bound to the ER (endoplasmic reticulum), where their regulatory domain co-localizes with an ER-embedded protein, SCAP (SREBP-cleavage-activating protein). SCAP functions as a sensor of membrane cholesterol levels and as an escort protein that promotes clustering of SREBPs in COPII (coat protein complex II)-coated membrane vesicles.

When cells become depleted in cholesterol, the SREBP–SCAP complex binds to COPII proteins and translocates from the ER to the Golgi, where a two-step proteolytic cleavage releases the N-terminal half of SREBP, allowing its entry into the nucleus [10]. SREBPs bind to SRE (sterol-regulatory element) and E box sequences in the promoter region of genes involved in cholesterol and fatty acid biosynthesis.

SREBP-1a is a potent activator of all SREBP-responsive genes, including those mediating the synthesis of cholesterol, fatty acids and triacylglycerols. The role of SREBP-1c is more restricted than that of SREBP-1a. SREBP-1c preferentially enhances the transcription of genes required for fatty acid, but not cholesterol, synthesis. SREBP-1c, a major mediator of the lipogenic action of insulin in liver, also activates the expression of target genes involved in glycolysis [11,12]. In vitro and in vivo studies have suggested that SREBP-1c may also contribute to the regulation of glucose uptake and glucose synthesis [13,14]. When overexpressed in hepatocytes, SREBP-1c induces the expression of glucokinases, key enzymes in glucose utilization, and suppresses phosphoenolpyruvate carboxykinase, a key enzyme of gluconeogenesis [13,14]. SREBP-1c expression is shown to be activated by LXR (liver X receptor), which forms heterodimers with RXR (retinoid X receptor) [15,16]. PUFAs (polysaturated fatty acids) suppress SREBP-1c transcription through an...
LXR-mediated mechanism [17]. Rodents fed on diets enriched with PUFAs manifest reduced SREBP-1c mRNA expression and low rates of hepatic lipogenesis [18]. In vitro, PUFAs inhibit SREBP-1c expression competitively, blocking LXR activation by its endogenous ligands. In addition to LXR-mediated transcriptional inhibition, PUFAs lower SREBP-1c levels by accelerating degradation of its mRNA [19]. Several lines of evidence show that the stimulatory effect of insulin on fatty acid synthesis is mediated by increasing SREBP-1c. In isolated rat hepatocytes, insulin treatment increases the amount of SREBP-1c mRNA in parallel with the mRNAs of its target genes. Conversely, incubating primary hepatocytes with glucagon decreases the mRNAs for SREBP-1c and its lipogenic target genes [20,21]. SREBP-1 is targeted by various post-translational modifications, including phosphorylation, acetylation, SUMOylation and ubiquitination [1].

It is well known that SREBP-1 expression is regulated by transcriptional, post-transcriptional (i.e. the turnover of SREBP-1 mRNA) and post-translational mechanisms. Translational regulation of SREBP-1 has not yet been studied. Therefore the aim of the present study was to investigate the role of human SREBP-1a and SREBP-1c 5′-UTRs (untranslated regions) on the translation efficiency of the SREBP-1 transcript. Results reported in the present paper demonstrated that, in Hep G2 and HeLa cells, the SREBP-1 5′-UTR strongly promotes the cap-independent translation of the downstream ORF (open reading frame) through an IRES (internal ribosome entry site).

Cellular stress, such as serum starvation, enhanced the precursor and the nuclear form of SREBP-1 in Hep G2 and HeLa cells. Therefore an IRES-mediated translation may account for the increase in the SREBP-1 protein level observed in serum-starved cells.

The UPR (unfolded protein response) pathway, a signalling cascade initiated by three ER-membrane-bound transducers, either facilitates the restoration of balance between ER load and capacity or promotes cell death. It has been shown that UPR activation triggers the proteolytic cleavage of SREBP-1c and the nuclear form of SREBP-1 in Hep G2 and HeLa cells. Therefore an IRES-mediated translation may account for the increase in the SREBP-1 protein level observed in serum-starved cells.

Cell culture and transient transfection assay

Hep G2 and HeLa cells were maintained in DMEM (Dulbecco’s modified Eagle’s medium) (Sigma) supplemented with 10% (v/v) heat-inactivated FBS (fetal bovine serum), penicillin G (100 units/ml) and streptomycin (100 μg/ml). Cells were kept at 37°C in a humidified atmosphere containing 5% CO2. For transient transfections, 5 × 104 cells were seeded into 12-well plates 48 h before transfection. Cells were transfected using FuGENE 6 (Roche Diagnostics) following the manufacturer’s recommendations. After an 8-h transfection period, the medium was changed to fresh DMEM supplemented with 10% (v/v) FBS and cells were incubated for 24 h. After cells lysis, RL (Renilla luciferase) and FL (firefly luciferase) activities were measured using the Dual Luciferase Reporter Assay System (Promega). The β-galactosidase activity was determined using a β-galactosidase assay. To study the effect of serum starvation, cells were maintained in DMEM supplemented with 0.5% FBS and incubated for 24 h.

[35S]Methionine incorporation

Hep G2 cells were plated at a density of 5 × 104 cells per 12-well dish and incubated for 48 h. [35S]Methionine/cysteine (30 μCi/ml; PerkinElmer) was then added to fresh medium and cells were incubated for a further 24 h in either serum-starved medium (0.5% FBS) or serum-supplemented medium [10% (v/v) FBS]. [35S]Methionine/cysteine incorporation was determined as reported previously [24].

Mono- and di-cistronic constructs

The 5′-UTR of the human SREBP-1a mRNA (GenBank® accession number NM_001005291) and SREBP-1c mRNA (GenBank® accession number AK293795) (Figure 1A) were amplified from total RNA by qRT-PCR (quantitative real-time PCR). Primers used in the PCR are listed in Table 1. The identity of the amplifiers was checked by DNA sequencing. The SREBP-1a 5′-UTR and SREBP-1c 5′-UTR amplifiers were then digested with HindIII and NcoI and inserted into the pGL3prom vector (Promega) to obtain pGL3S1a and pGL3S1c respectively (Figure 1B). For engineering the pBKLuc construct, the DNA fragment containing the FL coding region with its 5′ leader was excised from the pGL3prom by digestion with HindIII and XbaI, and then cloned into the pBluescriptII plasmid. Analogously, DNA fragments containing the SREBP-1a 5′-UTR and SREBP-1c 5′-UTR, followed by the FL coding region, were excised from pGL3S1a and pGL3S1c by digestion with HindIII and XbaI, and then cloned into the same sites of pBlueScriptII to obtain pBK51aLuc and pBKS1cLuc, respectively.

The plasmids pGL3-c-myc, pRF (formerly pGL3R), pHRF, pRe-mycF and pHpRe-mycF (formerly pGL3utrH) have been described previously [25] and were kindly provided by Dr A.E. Willis (Centre for Biomolecular Sciences, School of Pharmacy, University of Nottingham, Nottingham, U.K.). The SREBP-1a 5′-UTR and SREBP-1c 5′-UTR amplifiers were digested with EcoRI and NcoI and then inserted either into the intercistronic region of pRF or into the intercistronic region of pHpRF, in order to produce the dicistronic constructs pRS1aF and pRS1cF, or pHpRS1aF and pHpRS1cF respectively (Figure 1B).

To obtain promoterless dicistronic constructs, the SV40 (simian virus 40) promoter sequence, including the chimaeric intron between Smal and EcoRV sites, was removed by restriction

Table 1 Oligonucleotides used for qRT-PCR analysis and for construction of monocistronic and dicistronic vectors

<table>
<thead>
<tr>
<th>Primer Oligonucleotide sequence</th>
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<tr>
<td>SREBP-1rTFor 5′-ACACCATGCGGGAAGCACAC-3′</td>
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<tr>
<td>SREBP-1rTRev 5′-CTTCACTTCTACATGCTGCC-3′</td>
</tr>
<tr>
<td>hFASN rTFor 5′-GAAGAGGTGTTGTTCTGCC-3′</td>
</tr>
<tr>
<td>hFASN rTRev 5′-GGAAGACGACGTCTGACG-3′</td>
</tr>
<tr>
<td>hACACA rTFor 5′-ACACCAAGTAGTGAGGATG-3′</td>
</tr>
<tr>
<td>hACACA rTRev 5′-CTGTTGGAGATGAGTGAA-3′</td>
</tr>
<tr>
<td>rRNA18SFor 5′-GGTGGTTCTGAGAAGAAGG-3′</td>
</tr>
<tr>
<td>rRNA18Srev 5′-CGCAGCCTAGCTTTAGG-3′</td>
</tr>
<tr>
<td>S1a For1 5′-GAAGCTTAAATCCGGCGGGGAAACCAGT-3′</td>
</tr>
<tr>
<td>S1a For2 5′-GAATTCATGGGGAAGCACAC-3′</td>
</tr>
<tr>
<td>S1a Rev 5′-AAGCTTGAATTCTCGCCCTTCG-3′</td>
</tr>
<tr>
<td>S1c For1 5′-GAAGCTTGAATTCTCGCCCTTCG-3′</td>
</tr>
<tr>
<td>S1c For2 5′-GAATTCATGGGGAAGCACAC-3′</td>
</tr>
<tr>
<td>S1c Rev 5′-GAATTCATGGGGAAGCACAC-3′</td>
</tr>
<tr>
<td>iXBP1 For 5′-AGTGCGGCGTCTGCTGATG-3′</td>
</tr>
<tr>
<td>iXBP1 Rev 5′-CAAGATTCTGCAAGATCCCA-3′</td>
</tr>
</tbody>
</table>
After incubation for 2 h at 37°C, RNA was isolated and used to prime a 12.5 μl in vitro translation reaction mixture containing 8.25 μl of rabbit reticulocyte lysate (Promega) and up to 50 ng of RNA, as recommended in the manufacturer’s instructions.

**In vitro run-off transcription and translation**

pBKLuc, pBS1aLuc and pBS1cLuc were linearized at an XbaI site downstream of the FL ORF. Capped transcripts were synthesized in a reaction mixture containing 1 μg of DNA template and 20 units of T3 RNA polymerase (Promega), 1 mM ATP, 1 mM UTP, 1 mM CTP, 0.5 mM GTP, 2 mM m²G(5')ppp(5')G (Promega) and 20 units of RNasin in a final volume of 20 μl. For analysis of the human XBP1 (X-box-binding protein 1) mRNA splicing, cDNA was amplified with specific primers for the human XBP1 (X-box-binding protein 1) mRNA splicing, of FL mRNA was as described previously [25].

**Isolation of RNA, qRT-PCR and Northern blotting analysis**

Total RNA extraction from cultured cells and qRT-PCR analysis were carried out as described previously [26]. The amount of SREBP-1, FASN (fatty acid synthase) and ACACA (acetyl-CoA carboxylase α) mRNA was normalized to the internal control 18S rRNA. The sequence of primers used in the qRT-PCR analysis is shown in Table 1. Northern blotting analysis was carried out as described previously [27].

**Immunoelectrophoretic analysis**

Western blot analysis was carried out as reported previously [28]. After electrophoretic transfer of proteins to nitrocellulose, the blots were probed with antibody directed against SREBP-1 (Santa Cruz Biotechnology). The detection system employed was the ECL Plusμm Western Blotting Reagents (GE Healthcare).

**SREBP-1 half-life analysis**

Hep G2 cells were plated at a density of 1×10⁶ cells into 25 cm² flasks and incubated for 48 h. Cells were incubated for further 24 h in either serum-starved medium (0.5% FBS) or serum-supplemented medium (10% v/v FBS). Then, 100 μg/ml cycloheximide, an inhibitor of protein synthesis, was added to the medium and cells were incubated for the times indicated. At different times, cells from a flask were harvested and Western blot analysis was performed as described above. Autoradiograms were quantified by densitometric scanning.

**RESULTS**

**Human SREBP-1a and SREBP-1c 5'-UTRs inhibit the in vitro translation of the luciferase reporter gene**

Human SREBP-1a and SREBP-1c 5'-UTRs (185 bp and 94 bp respectively; Figure 1A) were inserted into the pBlueScripitll vector upstream of the FL start codon to obtain pBS1aLuc and pBS1cLuc respectively (Figure 1B). The control pBKLuc construct was generated by inserting the FL ORF with its 5' leader sequence, excised from the pGL3prom vector, into the pBlueScripitll vector. Capped mRNAs generated from these constructs by T3 RNA polymerase were then used to prime rabbit reticulocyte lysates and the FL activity was measured. We found that SREBP-1a and SREBP-1c 5'-UTRs strongly inhibited the translation of the downstream FL ORF. In fact, with pBS1aLuc and pBS1cLuc the FL activity fell by as much as 10- and 3-fold respectively, when compared with that measured by using the control pBKLuc (Figure 2A).
Does the SREBP-1a or SREBP-1c 5'-UTR contain an IRES?

The discrepancy between the results of the translation obtained in Hep G2 cells and those obtained in in vitro experiments led us to speculate that the SREBP-1a and SREBP-1c 5'-UTRs could contain an IRES. To test this hypothesis, SREBP-1a and SREBP-1c 5'-UTRs were inserted into the dicistronic vector pRF [25]. This plasmid contains two reporter genes. The first cistron (RL) is under the control of the SV40 promoter and it is translated via a cap-dependent mechanism, whereas the second FL cistron is translated independently from the cap structure [25, 29]. The SREBP-1a and SREBP-1c 5'-UTRs were cloned upstream of the FL cistron to obtain pRS1aF and pRS1cF respectively (Figure 1B). Hep G2 cells were co-transfected with either pRF, pRS1aF or pRS1cF, together with the pcDNA3.1/HisB/lacZ control plasmid. At 24 h after transfection, both RL and FL activities were measured and normalized to the transfection control β-galactosidase. The presence of the SREBP-1 5'-UTR between the two reporter genes did not alter RL activity (Figure 3A). Results reported in Figure 3(B) showed that, in the Hep G2 cells transfected with pRS1cF, the FL activity was approx. 13-fold higher than that determined with the pRF control plasmid, whereas, with pRS1aF, the FL activity was approx. 20-fold higher than that measured with the control pRF. The c-Myc 5'-UTR, inserted upstream of the FL cistron to obtain pRc-mycF, used as a positive control, stimulated the expression of the downstream cistron by approx. 50-fold when compared with the pRF control plasmid, in agreement with a previous report [25]. Several control assays were performed to determine whether this result might be ascribed to mechanisms alternative to IRES, such as enhanced ribosomal reinitiation at the FL start codon, and/or generation of FL mRNA either by differential splicing or from a cryptic promoter. In order to investigate the ribosomal reinitiation mechanism, SREBP-1a and SREBP-1c 5'-UTRs were cloned into the pHpRF vector to obtain the pHpRS1aF and pHpRS1cF constructs respectively (Figure 1B). These constructs contain, upstream of the RL coding region, an inverted repeat sequence, which produces a stable hairpin structure in the mRNA (−55 kcal/mol). Cap-dependent translation of the upstream RL cistron should be greatly reduced, whereas cap-independent translation of the downstream FL cistron should not be affected. As expected, the pHpRS1aF and pHpRS1cF constructs containing the hairpin structure showed a reduction in RL activity in Hep G2 cells by approx. 80% when compared with pRF control (Figure 3C). On the contrary, with both the constructs, an FL activity higher by ∼20- and ∼12-fold respectively, when compared with the pRF plasmid control, was observed (Figure 3D).

The enhanced expression of the downstream FL ORF might be ascribed to the translation of a shorter monocistronic transcript derived from a cryptic promoter in the 5'-UTR of the pRS1aF or pRS1cF constructs. To assess this hypothesis, the promoterless pRF(-p), pRS1aF(-p) and pRS1cF(-p) constructs were obtained by removing the SV40 promoter from pRF [30], pRS1aF and pRS1cF (Figure 1B). As shown in Figure 3(E), transfection with the promoterless pRS1aF(-p) resulted in minimal luciferase activity in Hep G2 cells, indicating that the FL expression from this construct did not depend on a cryptic promoter activity. On the other hand, after transfection of pRS1cF(-p), FL activity was approx. 5-fold higher than that determined with the pRF(-p) control. This result suggested that a cryptic promoter was created when the SREBP-1c 5'-UTR was inserted upstream of the FL cistron.

To investigate whether a FL monocistronic transcript originated by alternative splicing from dicistronic RNA, Northern blot analysis was carried out, using the FL ORF as a probe. In Hep G2 cells.
cells transfected with pRF or pRS1αF, a dicistronic transcript was detected (Figure 3F, lanes 2 and 3).

Notably, two strong autoradiographic signals were observed in Hep G2 cells transfected with pRS1αF, the first corresponding to a dicistronic RNA and the second corresponding to a shorter transcript, probably containing only the FL cistron (Figure 3F, lane 4). These results are indicative of the presence of a cryptic promoter sequence in the SREBP-1c 5′-UTR. The autoradiographic signals of the two transcripts were similar, indicating that the cryptic promoter exhibits quite strong activity.

IRES activity during serum deprivation

Cellular stress, such as serum, amino acid and glucose starvation, heat shock, oxygen deprivation, apoptosis and mitosis, is known to inhibit cap-dependent, but not cap-independent, translation [31]. As a consequence, translation of mRNAs harbouring an IRES in their 5′-UTR ensures adequate protein synthesis, through the cap-independent mechanism, even when the global translation is reduced. To investigate whether serum starvation could affect SREBP-1 translation, the protein levels of the pSREBP-1 and nSREBP-1 forms of SREBP-1 were quantified in control and serum-starved Hep G2 cells. Western blotting experiments showed that pSREBP-1 and nSREBP-1 levels increased approx. 2-fold in Hep G2 cells after 24 h of serum deprivation when compared with the control cells (Figure 4A). In contrast, serum deprivation did not cause a significant variation in SREBP-1 mRNA levels in starved compared with control Hep G2 cells (Figure 4B). Similar results were obtained in HeLa cells (Figures 4A and 4B). To address the question of whether serum deprivation affects protein synthesis, incorporation of [35S]methionine was measured in starved and control Hep G2 cells. As shown in Figure 4(C), the incorporation of [35S]methionine decreased by approx. 60% in starved cells compared with control cells.

To investigate whether serum deprivation enhances protein stability, the half-life of pSREBP-1 and nSREBP-1 was determined in starved and control Hep G2 cells. The log of pSREBP-1 and nSREBP-1 content was reported as a function of time (Figure 4D). Results show an increase in the turnover of pSREBP-1 in starved cells compared with control cells. The apparent half-life of pSREBP-1 protein was ∼2.5 h in starved cells compared with ∼6.5 h in control cells. In contrast, the apparent half-life of nSREBP-1 was slightly lower in starved cells compared with control cells (∼5.2 h compared with ∼6.5 h respectively).

The turnover of pSREBP-1 and nSREBP-1 was also evaluated in Hep G2 cells cultured in the presence of 10 μg/ml cholesterol and 1 μg/ml 25-hydroxycholesterol, which block the proteolytic cleavage of pSREBP-1. Sterols were added to the medium 5 min prior the addition of cycloheximide. In this condition, the half-life of nSREBP-1 was strongly diminished in starved cells compared with control cells (∼1.6 h compared with ∼5.1 h respectively). In contrast, the decay curve of pSREBP-1 was similar in starved cells and in control cells (Figure 4D).
Figure 4  Effect of serum starvation on the SREBP-1 protein level

(A) Hep G2 cells were incubated for 24 h in DMEM with either 10% (v/v) or 0.5% FBS. Cells were then harvested for preparation of a crude nuclear fraction. Proteins (50 μg) were separated by SDS/PAGE and immunoblotted with antisera against SREBP-1. The content of pSREBP-1 and nSREBP-1 in cells cultured in either serum-starved medium (0.5% FBS) or serum-supplemented medium (10% (v/v) FBS) was analysed by Western blotting, quantified by densitometric analysis and is expressed as the fold change relative to SREBP-1 content in control cells cultured in serum-supplemented medium. Values are means ± S.D., n = 4. (B) Total RNA was extracted from Hep G2 cells cultured in either serum-starved medium or serum-supplemented medium for 24 h. SREBP-1 mRNA level was determined by using qRT-PCR and normalized with 18S rRNA. Values are reported as the fold change relative to control. (C) At 48 h after cell plating, L-[35S]methionine/cysteine was added to fresh medium and cells were incubated for further 24 h in either serum-starved medium or serum-supplemented medium. [35S]Methionine/cysteine incorporation was normalized with respect to total protein determined using the Bradford assay. Values are reported as the fold change relative to control cells cultured in serum-supplemented medium. For (B and C), values are means ± S.D. (n = 4). (D) Hep G2 cells, incubated for 24 h in DMEM with either 10% (v/v) or 0.5% FBS, were then treated with 100 μg/ml cycloheximide. At different times, cells were harvested and the content of either pSREBP-1 or nSREBP-1 protein was measured by Western blot analysis. The semi-log plot represents the decay curve of pSREBP-1 (squares) or nSREBP-1 (circles) protein in control (filled) and serum-starved (open) Hep G2 cells, incubated in the absence (left-hand panel) or in the presence (right-hand panel) of sterols. The results are from a representative experiment, with similar results being obtained in four independent experiments.

The increase in SREBP-1 protein levels observed in starved Hep G2 or HeLa cells could be ascribed to an efficient translation of its mRNA through an IRES-mediated mechanism. As the insertion of SREBP-1c 5′-UTR in the dicistronic pRF vector resulted in cryptic promoter activity (Figures 3C and 3F), the observed increase in FL activity might be ascribed, at least in part, to an augmentation of the FL transcript abundance. However, we cannot rule out that an IRES is also present in the SREBP-1c 5′-UTR and that it could contribute to an increase in SREBP-1 protein levels.

The IRES in the SREBP-1a 5′-UTR might allow efficient translation of its mRNA during cellular stress, such as serum starvation.
independent experiments. Values are means + − standard deviation of triplicate samples.

Figure 5  IRES activity is maintained upon serum starvation

Hep G2 cells were transiently transfected with pRS1aF and pRc-mycF. After transfection, cells were subjected to serum starvation or were left under control conditions for a further 24 h before harvesting and performing assays for RL and FL activities. Values for RL and FL activities measured in cells transfected with pRS1aF (A) or pRc-mycF (B) and cultured in serum-starved medium (0.5% FBS) are reported as the percentage of the corresponding luciferase activities determined in cells transfected with the same constructs and cultured in serum-supplemented medium (10% (v/v) FBS). Values are means ± S.D. of triplicate samples from each of five independent experiments.

starvation. If this were the case, translation of FL from the dicistronic pRS1aF mRNA would be relatively unaffected by serum starvation. To test this hypothesis, Hep G2 cells transfected with the pRS1aF were subjected to serum starvation for 24 h and then the luciferase activity was measured (Figure 5). Upon serum starvation, RL activity was reduced by approx. 70% compared with RL activity measured in cells cultured in serum-supplemented medium (Figure 5A). This is consistent with the effect of serum starvation on the total translation rate measured by [35S]methionine incorporation in cultured cells (Figure 4C). In serum-starved cells, FL activity was reduced by 33% compared with that measured in control cells (Figure 5A). Similar results were obtained when transfecting the same cells with pRc-mycF, containing the c-Myc 5′-UTR upstream of the FL ORF (Figure 5B).

Translation from the SREBP-1a IRES is stimulated upon ER stress

Next, we investigated whether the UPR response also stimulates translation from the SREBP-1a IRES. ER stress in Hep G2 cells was induced by thapsigargin or tunicamycin. Thapsigargin modifies Ca2+ concentrations in the ER lumen by inhibiting Ca2+-ATPase. Tunicamycin leads to accumulation of proteins into the lumen of the ER by inhibiting protein glycosylation. In agreement with previous studies [22,32], incubation for 1 h in the presence of 300 nM thapsigargin or 1 μg/ml tunicamycin induced the splicing of XBP1 mRNA, a classical index of the UPR (results not shown).

To evaluate the time-dependent effect of thapsigargin (300 nM) or tunicamycin (1 μg/ml) on the expression of SREBP-1 in HepG2 cells, Western blot analysis was carried out. Treatment of Hep G2 cells with thapsigargin for 15 min caused a decrease in pSREBP-1 and an increase in nSREBP-1 (Figure 6A). However, after incubation for 1 h with thapsigargin, an increase in both pSREBP-1 and nSREBP-1 was observed, rising up to a maximum level at 3 h (Figure 6A). Addition of tunicamycin to Hep G2 cells caused a stronger activation of both pSREBP-1 and nSREBP-1 expression than that observed in thapsigargin-treated cells, reaching a maximum level at 6 h of treatment (Figure 6A). The mRNA level of SREBP-1 was unchanged in tunicamycin-treated cells when compared with control cells (Figure 6B).

The effect of SREBP-1 activation on the expression of its target lipogenic genes, i.e. FASN and ACACA, was also analysed. Hep G2 cells were cultured for 1, 3, 6, 12 and 24 h in DMEM in the presence of tunicamycin, which induced an increase in the expression of FASN and ACACA (Figure 6B). Similar results were obtained in thapsigargin-treated cells (results not shown).

To test the effect of ER stress on translation from the SREBP-1a IRES, Hep G2 cells, transiently transfected with pRS1aF, were treated with thapsigargin or tunicamycin (Figure 6C). Both ER stressors already decreased RL activity after 15 min. This effect was consistent with the inhibition of cap-dependent translation by these agents. In contrast, both treatments caused an increase in FL activity, reaching the maximum level at 6 h of treatment (Figure 6C).

To determine whether ER stress affects protein stability, the half-life of pSREBP-1 and nSREBP-1 was determined in tunicamycin-treated Hep G2 cells. Results show that tunicamycin reduced the half-life of pSREBP-1 (Figure 6D) when compared with control cells (∼1.9 h compared with ∼6.5 h respectively) (Figure 4D). No significant change in the half-life of nSREBP-1 was observed in tunicamycin-treated cells (Figure 6D) when compared with control cells (Figure 4D) (∼6.3 h compared with ∼6.5 h in control cells). Addition of sterols to the medium reduced the turnover of pSREBP-1 in tunicamycin-treated cells when compared with the same cells without sterols (Figure 6D) (half-life of ∼5.9 h in cells with sterols compared with ∼9.9 h in cells without sterols). In contrast, the half-life of nSREBP-1 strongly diminished in tunicamycin-treated cells incubated with sterols when compared with that observed in the same cells without sterols (∼2.3 h in cells with sterols compared with ∼6.3 h in cells without sterols).

DISCUSSION

Translational control is a final regulatory step in gene expression. In the ribosome scanning model of translation [33], the 5′-end m′G structure of mRNA is recognized by the cap-binding protein complex eIF (eukaryotic initiation factor) 4F. The binding of the eIF4F complex to mRNA further recruits other initiation factors as well as the 40S ribosomal subunit. This complex proceeds in the 3′ direction until an AUG start codon in a favourable context is encountered and protein synthesis is initiated.

A broad range of cellular stresses lead to the inhibition of translation. This event is accomplished by the phosphorylation of some initiation factors and/or their regulators [34] or by the proteolytic cleavage of several initiation factors [35].

Under conditions of reduced translation, mRNAs encoding for several oncoproteins, survival factors and proteins critically involved in apoptosis are translated by a poorly understood cap-independent mechanism [36]. This mechanism is mediated by IRES elements found in the 5′-UTR of a limited, but growing, number of mRNAs preferentially involved in the control
of cellular proliferation, survival and death (for reviews see [37–39]).

There is a growing interest among researchers about SREBP-1, due to the important role that this protein exerts on lipid homeostasis in the organism. Regulation of SREBP-1 expression is very complex and involves several steps at transcriptional, post-transcriptional and post-translational levels. Among post-transcriptional mechanisms, no results are available for SREBP-1 regulation at translational level.

In the present study, the characterization of the SREBP-1α and SREBP-1c 5′-UTRs has been reported, showing that an IRES element is present in the 5′-UTR of SREBP-1 mRNA.

In vitro translation experiments carried out using rabbit reticulocyte lysates demonstrated that SREBP-1α 5′-UTR and, to a lesser extent, SREBP-1c 5′-UTR, inhibited the translation of the downstream FL ORF (Figure 2A). This finding suggests that a stable secondary structure could obstruct the scanning translation initiation complex or that non-canonical factors, absent in rabbit reticulocyte lysates, were required for in vitro translation initiation of SREBP-1 mRNAs [40]. On the basis of structure-prediction algorithms mfold [41], both the human SREBP-1α and SREBP-1c 5′-UTRs, characterized by a high GC percentage (approx. 79 and 71% respectively), contain extensively RNA secondary structures, with those of the SREBP-1α 5′-UTR being more complex than those of the SREBP-1c 5′-UTR (results not shown).

Experiments performed either with monocistronic pGLS1α and pGLS1c or with dicistronic pRS1αF and pRS1cF constructs demonstrated that both SREBP-1α and SREBP-1c 5′-UTRs did not inhibit, but augmented, FL expression when compared with the controls (Figures 2B and 3B). Moreover, the palindromic sequence inserted into pHpRS1αF and pHpRS1cF constructs forms a stable RNA hairpin, which reduced RL activity by 80%, but did not affect FL activity (Figures 3C and 3D). If a ribosomal readthrough mechanism, caused by the 5′-UTR insertion, was responsible for the translation of FL, then this activity should be reduced by a similar value. Moreover, transfection experiments performed with the promoterless pRS1αF(-P) construct demonstrated that the enhanced expression of FL driven by SREBP-1α 5′-UTR cannot be ascribed to a cryptic promoter activity, as FL activity drastically fell (Figure 3E). In contrast, after transfection with the promoterless pRS1cF(-P) construct, the expression of FL was 5-fold higher than that measured in cells transfected with the pRF(-P) control plasmid.
(Figure 3E). Therefore enhanced FL expression promoted by SREBP-1c 5′-UTR could be due, at least in part, to a cryptic promoter activity. The presence of a cryptic promoter has also been reported by Han and Zhang [30] in the 5′-UTR of eIF4G mRNA. Northern blot analysis confirmed this finding, as a short transcript (Figure 3F, lane 4), probably containing only the FL ORF, was observed in the cells transfected with pRS1cF besides the dicistronic RNA, whereas, in cells transfected with pRS1aF, only the long transcript was detected (Figure 3F, lane 3). Taken together, these findings, for the first time, support the notion that the SREBP-1a 5′-UTR is capable of internal translation initiation. However, we cannot rule out that an IRES element is also present in the SREBP-1c 5′-UTR. Indeed, FL activity measured in Hep G2 cells transfected with the dicistronic pRS1cF construct was 12-fold higher than that determined with the pRF control vector (Figure 3B) and, thus, approx. 2.4-fold higher than that observed in transfection experiments carried out with the promoterless construct pRS1cF(-P) (Figure 3E).

In the present study, we showed that a cellular stress condition, such as serum starvation, caused an increase in the level of SREBP-1 precursor and mature form in both Hep G2 and HeLa cells (Figure 4A), despite the overall reduction in protein synthesis, as demonstrated by the decrease in [35S]methionine incorporation (Figure 4C). Furthermore, no significant increase in the amount of SREBP-1 mRNA was observed in starved Hep G2 or HeLa cells (Figure 4B). On this basis, we speculate that, in a cellular stress condition, the increase in SREBP-1 content could be due to an enhanced SREBP-1a mRNA translation through an IRES-mediated mechanism.

This hypothesis is supported by results on the RL and FL activities measured in control and in starved Hep G2 cells transfected with the dicistronic construct pRS1aF (Figure 5). Indeed, although serum starvation reduced both RL and FL activities, the former was affected more, suggesting that IRES-dependency was less sensitive to serum starvation than cap-dependent translation (Figure 5). The change in SREBP-1 content observed in starved Hep G2 cells might be ascribed to an increase in protein stability rather than to the IRES mechanism proposed. To address this question, the half-life of pSREBP-1 and nSREBP-1 was evaluated in starved and control Hep G2 cells. Results showed that the turnover of pSREBP-1 strongly increased in starved cells when compared with control cells (Figure 4D). Note that the half-life of nSREBP-1 was affected to a lesser extent than that of pSREBP-1 by serum starvation (Figure 4D). The reduction in pSREBP-1 content observed in starved Hep G2 cells could be due to an increase in its proteolytic cleavage rather than to its degradation. Therefore we evaluated the turnover of pSREBP-1 and nSREBP-1 in starved and in control Hep G2 cells cultured in the presence of cholesterol and 25-hydroxycholesterol, which block the proteolytic cleavage of pSREBP-1. In starved Hep G2 cells, the turnover of pSREBP-1 decreased in the presence of sterols when compared with that observed in starved cells cultured without sterols. In contrast, the half-life of nSREBP-1 strongly diminished in starved cells cultured in the medium with sterols added (Figure 4D) when compared with the same cells cultured in the absence of sterols. In control Hep G2 cells, the turnover of nSREBP-1 and pSREBP-1 was unaffected by the addition of sterols, ruling out the hypothesis of a direct role of sterols in altering the SREBP-1 stability. Taken together, these results show that serum starvation: (i) induces the proteolytic cleavage of SREBP-1, and (ii) enhances SREBP-1 turnover.

A number of reports have highlighted the link between the UPR and hepatic lipid metabolism [42,43]. It has been shown that the homocysteine- or thapsigargin-induced UPR was able to activate SREBP-1c and to induce lipogenic gene expression by promoting the proteolytic cleavage of pSREBP-1 [22,44]. We observed a similar effect on ER-stressed Hep G2 after incubation in thapsigargin- or tunicamycin-supplemented medium (Figure 6A). A strong increase in both pSREBP-1 and nSREBP-1 content was observed after 1 h of treatment with the ER stressors (Figure 6A). The increase in SREBP-1 content observed in ER-stressed Hep G2 cells could be ascribed to an enhanced SREBP-1 mRNA translation through a cap-independent mechanism, as demonstrated by transfection experiments with the dicistronic construct pRS1aF (Figure 6C). As the half-life of both pSREBP-1 and nSREBP-1 decreased in ER-stressed Hep G2 cells when compared with control cells, it could be ruled out that an altered protein stability caused the increase in SREBP-1 content observed in ER-stressed cells (Figure 6D).

The physiological role of the IRES in the SREBP-1a 5′-UTR could be to allow minimal lipogenic and cholesterologenic activities, even when cap-dependent translation is inhibited. Protein synthesis, via internal ribosome entry, may be required in several conditions such as mitosis, where cap-dependent translation is reduced [45,46]. It has been reported that the mature forms of SREBP-1a and SREBP-1c are hyperphosphorylated in mitotic cells [47], and Cdk1 (cyclin-dependent kinase 1)/cyclin B-mediated phosphorylation is important for their stabilization during mitosis [48]. On the basis of our present results, we speculate that enhanced translation of SREBP-1 through the cap-independent mechanism could work synergistically with Cdk1/cyclin B-mediated phosphorylation and stabilization of SREBP-1, in order to promote lipid and cholesterol synthesis during mitosis. This hypothesis is in agreement with the observation that siRNA (small interfering RNA)-mediated knockdown of SREBP-1 resulted in an increase in cells in G1-phase accompanied by a decrease in cells in S-phase, caused by an impaired ability to proliferate [47].

Deletion or site-specific mutation analysis of SREBP-1a 5′-UTR will be performed to characterize the minimal region containing the IRES, together with the ITAFs (IRES transactivating factors) mediating the cap-independent translation of SREBP-1 upon serum starvation or ER stress.

AUTHOR CONTRIBUTION

Fabrizio Damiano designed and performed the experiments, analysed the data and contributed to the writing of the manuscript; Simone Alemanno performed the experiments; Gabriele Groni obtained financial support, directed the study and helped edit the manuscript; Luisa Siculella helped in scientific discussions, and wrote and edited the manuscript. All the authors discussed the results and commented on the paper.

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