Phosphorylation of initiation factor-2α is required for activation of internal translation initiation during cell differentiation

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The long uORF-burdened 5’UTRs of many genes encoding regulatory proteins involved in cell growth and differentiation contain internal ribosomal entry site (IRES) elements. In a previous study we showed that utilization of the weak IRES of platelet-derived growth factor (PDGF) is activated during megakaryocytic differentiation. The establishment of permissive conditions for IRES-mediated translation during differentiation has been confirmed by our demonstration of the enhanced activity of vascular endothelial growth factor, c-Myc and encephalomyocarditis virus IRES elements under these conditions, although their mRNAs are not naturally expressed in differentiated K562 cells. In contrast with the enhancement of IRES-mediated protein synthesis during differentiation, global protein synthesis is reduced, as judged by polysomal profiles and radiolabelled amino acid incorporation rate. The reduction in protein synthesis rate correlates with increased phosphorylation of the translation initiation factor eIF2α. Furthermore, IRES use is decreased by over-expression of the dominant-negative form of the eIF2α kinase, PKR, the vaccinia virus K3L gene, or the eIF2α-S51A variant which result in decreased eIF2α phosphorylation. These data demonstrate a connection between eIF2α phosphorylation and activation of cellular IRES elements. It suggests that phosphorylation of eIF2α, known to be important for cap-dependent transaltional control, serves to fine-tune the translation efficiency of different mRNA subsets during the course of differentiation and has the potential to regulate expression of IRES-containing mRNAs under a range of physiological circumstances.

Keywords: differentiation; gene expression; initiation factor 2; IRES; translation initiation.

Translation of eukaryotic gene expression is controlled both by global mechanisms that affect the overall rate of protein synthesis and by selective control mechanisms that affect the translation of subsets of mRNA molecules equipped with appropriate cis-regulatory elements. The global mechanisms are mostly based on controlling the availability of two rate-limiting components of the initiation step: eIF4E, the 5’-cap binding protein, and eIF2, a GTP-binding protein that mediates the association of Met-tRNAi to the 40 S ribosomal subunit. Control of eIF4E activity is mediated by influencing its phosphorylation status and/or its interaction with the eIF4E binding proteins, as well as by affecting the integrity of eIF4G which serves as an adapter protein that bridges eIF4E, the RNA helicase eIF4A, poly(A) binding protein and eIF3 (reviewed in [1–3]). Control of eIF2 activity is mediated through reversible phosphorylation of its α-subunit. When eIF2α is phosphorylated, the GDP-eIF2 generated at the end of each initiation step becomes a competitive inhibitor of eIF2B, a rate-limiting guanine nucleotide exchange factor, resulting in a reduction of the exchange of eIF2-bound GDP for GTP. As GTP binding to eIF2 is a prerequisite to Met-tRNAi binding, phosphorylation of eIF2α effectively inhibits eIF2 recycling and consequently inhibits additional translation initiation steps (reviewed in [4]). The control mechanisms that govern the rate of global protein synthesis are responsive to a variety of conditions including nutrient deprivation, heat shock, apoptosis and viral infection. Under conditions that inhibit the initiation of global protein synthesis, subsets of mRNAs remain competent to be recruited by ribosomes. Depending on their specific cis-regulatory elements they may gain a translational advantage over other mRNA molecules. For instance, mRNAs encoding heat-shock proteins are translated efficiently under conditions of reduced eIF4E/4F activity due to their unstructured 5’UTR (reviewed in [5]). Another example is the efficient translation of the yeast GCN4 mRNA under conditions of amino acid starvation due to leaky scanning of the upstream ORF within its 5’UTR (reviewed in [6]).

While much data has been accumulated regarding the control of protein synthesis in response to various stress conditions, less is known about translational control mechanisms that are operative during cellular differentiation. Cells undergoing terminal differentiation exhibit extensive changes in the pattern of gene expression to acquire a specific biological function that is usually accompanied by cessation of proliferation. In addition to the massive changes at the transcriptional level, mechanisms regulating overall inhibition of protein synthesis release most mRNAs from the polysomes and facilitate the translation of specific mRNAs that are important for the
phosphorylation of eIF2 during differentiation and to ascertain whether eIF2 remained of interest to determine eIF2 protein, eIF4E, during differentiation. However, it is important for cap-dependent translational control; and (c) synthesis rate is reduced during differentiation, correlating with increased IRES elements, although their mRNAs are not naturally expressed in differentiated K562 cells; (b) global protein synthesis rate is reduced during differentiation, correlating with increased eIF2α phosphorylation that is known to be important for cap-dependent translational control; and (c) inhibition of eIF2α phosphorylation during differentiation reduces the differentiation-induced IRES activation.

MATERIALS AND METHODS

Plasmids

The pLL vector is composed of a fragment containing Renilla luciferase from pRL-null (Promega) as the first cistron, fused to a pGL3-basic (Promega) as the second cistron. A 22-base pair fragment containing Stul and NcoI sites separates the stop codon of the Renilla luciferase and the ATG initiator codon of the firefly luciferase. An NheI (filled)–SacI fragment containing both cistrons was ligated with a HindIII (filled)–SacI fragment of pCL [12] to generate pLL, which contains the dual luciferase transcription unit downstream of the cytomegalovirus (CMV) promoter and upstream of the SV40 intron and polyadenylation sites. The 5'UTRs of VEGF and of PDGF2 were obtained as SpeI (filled)–NcoI fragments from pSKVLUC [16] and pCPL [12], respectively, and were ligated to the Stul–NcoI 7.5-kb fragment of pLL to generate pLVL and pPLL, respectively. The EMCV IRES fragment was obtained as Aho26I (filled)–NcoI fragment from pTM1 [17], and was ligated to the Stul–NcoI 7.5-kb fragment of pLL to generate pLEL. P2-c-Myc 5'UTR (Genebank accession # J00120) was generated by RT-PCR using total RNA from K562 cells and the oligonucleotides 5'-CCCCACTAGTAAATTCCAGCGAGAGGCAGA-3' and 5'-AATACCGGAGGCTGCTG-3', and was ligated to the Stul–NcoI 7.5-kb fragment of pLL to generate pLML. pcK3L was generated by insertion of the NcoI (filled)–BamHI 0.3-kb fragment of pTM1-K3L [18] into the HindIII (filled)–BamHI sites of pcDNA3 (Invitrogen) under the control of the CMV promoter. pcPKRA6 = p68Δ56-pcDNAI/NEO [19] was used for expression of PKRA6, the dominant-negative variant of PKR under the control of the CMV promoter. pEGFP-N3 plasmid (Clontech) expressing GFP under the control of the CMV promoter was used as control plasmid for the cotransfection experiments.

Cells and megakaryocytic differentiation

The human chronic myelogenous leukemia cell line K562 was grown in RPMI 1640 medium (Biological Industries) supplemented with 50 U penicillinmL^-1, 50 μg/mL streptomycin, 0.1 mg/mL kanamycin and 10% fetal bovine serum. Cells at a density of 5–7 × 10⁶ cellsmL^-1 or 1.2–1.5 × 10⁵ cellsmL^-1 were considered as logarithmically growing (log) or growth arrested (dense), respectively. Megakaryocytic differentiation was induced by dilution of cells at a density of 1.2 × 10⁶ cellsmL^-1, to a final concentration of 5 × 10⁵ cellsmL^-1, with medium containing 5 nm 12-O-tetradecanoylphorbol-13-acetate (TPA; Calbiochem) for 48 h.

Plasmid transfections and luciferase assays

Twenty or forty micrograms supercoiled DNA of each of the bi-cistronic vectors or the cotransfected plasmid, respectively, were used per 7.5 × 10⁵ K562 cells for each electroporation sample. Electroporation was performed in 0.8 mL RPMI 1640 without serum by an electric pulse of 240 V and 1500 mF (Easy Ject1 Electroporator; Equibio). Immediately following the electric pulse, the cells were transferred to 10 mL RPMI 1640 medium supplemented with 20% fetal bovine serum for 24 h. The cells were diluted to a final concentration of 5 × 10⁵ viable cellsmL^-1.
(as determined by Trypan blue staining) in RPMI 1640 supplemented with 10% fetal bovine serum, with or without 5 nM TPA, for 48 h. Transfection efficiency was 50–60%, as judged by the percentage of fluorescent GFP-expressing cells. The control and differentiated transfectants were harvested simultaneously, and assayed for Renilla and firefly luciferase activities using the Dual-luciferase reporter assay system (Promega) and TD-20e-Luminometer (Turner). RNA was isolated from cells transfected with the bicistronic constructs and analysed by Northern blotting using a LUC-specific probe to ensure that transcripts of the correct size were produced. Bi-cistronic transcript level from all constructs was approximately five times higher in differentiated cells because of the increased activity of the CMV promoter [12].

**32P labelling, immunoprecipitation and Western analysis of eIF2α**

A total of 10^6 log, dense or megakaryocytic differentiated K562 cells were washed twice with Heps/saline buffer (50 mM KOH/Heps pH 7.0, 150 mM NaCl) and resuspended in 0.5 mL Dulbecco’s modified Eagle medium lacking sodium phosphate (Sigma) supplemented with 10% dialysed fetal bovine serum. The cells were labelled for 2 h with 0.2 mCi [32P]Pi.mL^-1 (Amersham, #PBS13), followed by two washes with cold NaCl/Pi, containing 10 mM β-glycero phosphate and 50 mM β-glycerophosphate. Cells were extracted from the cell pellets by using 470 mL lysis buffer containing 25 mM KOH/Heps pH 7.2, 0.5% EluantTM (Calbiochem), 100 mM KCl, 0.05% SDS, 1 mM dithiothreitol, 2 μM okadaic acid, 10 mM β-glycerophosphate, 50 mM NaF and protease inhibitor cocktail (Complete™, Roche). For immunoprecipitation the sample was supplemented with 28 μL 5 mM NaCl and 0.5 μL anti-eIF2α mAb [21] and incubated for 1 h at 4°C. Next, rabbit anti-(mouse IgG) Ig (Jackson Immuno Research) were added for further incubation of 1 h, followed by addition of 10 μL packed protein A-Sepharose (Pharmacia Biotech) for an additional 1-h incubation. Following separation of the immunoprecipitate by SDS/10% PAGE, the proteins were blotted onto a nitrocellulose membrane and quantified by phosphoimager. The membrane was then used for Western analysis using antibodies specific for Ser51-phosphorylated eIF2α (Research Genetics, Inc.), and following stripping mAb specific for total eIF2α were used.

**Polysome fractionation**

A total of 3.5 × 10^7 log, dense, or megakaryocytic differentiated K562 cells were treated with 90 μg.mL^-1 cycloheximide for 10 min prior to harvest and used for fractionation of polysomes by sedimentation through 5-47% sucrose gradients [22].

**Protein synthesis rate**

One million log, dense, or megakaryocytic differentiated K562 cells were re-suspended in 2 mL RPMI medium containing 10% fetal bovine serum. The cells were labelled for 20 min with 20 μCi.mL^-1 [35S]methionine, [35S]cysteine mix (NEN, #NEG072), followed by two washes with cold NaCl/Pi. Proteins were extracted from the cell pellets using 50 μL lysis buffer containing 25 mM KOH/Heps pH 7.5, 1% Triton X-100, 100 mM KCl, 1 mM dithiothreitol, 2 μM okadaic acid, 10 mM β-glycerophosphate, 50 mM NaF and protease inhibitor cocktail (Complete™, Roche). Twenty micrograms total protein were applied onto 3 mm filter papers (Whatman) and washed three times for 1 min in boiling 5% (W/V) trichloroacetic acid containing traces of cold i-methionine and i-cysteine. The filters were then rinsed once in ethanol, dried and counted in a scintillation counter (Beckman).

**Cell cycle analysis and differentiation markers**

For cell cycle analysis 5 × 10^8 cells were harvested, washed with NaCl/Pi, and re-suspended in 0.5 mL NaCl/P, containing 0.1% sodium azide. Following addition of 50 μL NaCl/P containing 1% Triton X-100 and 50 μL 1 mg.mL^-1 propidium iodide, the cell-cycle of the cells was analysed by Becton Dickinson FACSort, using the Cell Quest software. The Vav protein was used as a marker for differentiation. To detect Vav protein level the cells were lysed using a buffer containing 25 mM KOH/Heps pH 7.5, 1% Triton X-100, 100 mM KCl, 1 mM dithiothreitol, 2 μM okadaic acid, 10 mM β-glycerophosphate, 50 mM NaF and protease inhibitors cocktail (Complete™, Roche). 140 μg of total cell proteins were separated by SDS/10% PAGE, and blotted onto a nitrocellulose membrane which was then used for Western analysis using polyclonal antibodies specific for Vav (Santa Cruz) and polyclonal antibodies specific for CKHx (a gift from D. Canani, Tel Aviv University, Israel).

**RESULTS**

Favorable conditions for IRES-mediated translation are established during differentiation

In previous studies we have demonstrated that in addition to transcriptional activation of PDGF2 during megakaryocytic differentiation, its IRES element undergoes functional activation during the differentiation process [12,13]. Interested in elucidating the mechanism of IRES function in general, we have used the differentiation phase to learn more about the possible involvement of trans-acting factors. Viewing the differentiated state as a ‘permissive’ environment for PDGF2-IRES mediated translation, we also wished to check the effect of differentiation conditions on the behaviour of additional cellular and viral IRES elements. Although normally the mRNAs of VEGF and c-Myc are not present in differentiated megakaryocytes, it was still of interest to check the activity of their IRES elements in the differentiated K562 cells that are permissive for PDGF2 IRES use. The IRES elements of human VEGF, human c-Myc and EMCV were cloned into a CMV promoter-driven bicistronic vector, between the coding regions of Renilla and firefly luciferases, as illustrated in Fig. 1. K562 cells were transfected with each of the recombinant plasmids followed by incubation under normal or differentiation conditions for 48 h prior to measurements of Renilla and firefly luciferase enzymatic activities. As shown in Table 1, in differentiated cells we observed elevation in the activity of both luciferases, because of increased CMV promoter activity in this system which results in a fivefold increase in transcript levels (demonstrated in Fig. 3 [12]). However, upon differentiation,
Reduction of global protein synthesis during differentiation is accompanied by eIF2α phosphorylation

The terminal differentiation process is usually accompanied by arrest of cellular proliferation and thereby decreased global protein synthesis [7,8]. As megakaryocytic differentiated cells cease to proliferate [23], we wished to check the status of their global mRNA translation. The rate of radio-labelled amino acids incorporation in logarithmically growing K562 cells was compared to that in density-arrested or differentiated cells. The incorporation rate was almost two times lower in both stationary (dense) and differentiated cells. The incorporation rate was almost two times lower in both stationary (dense) and differentiated cells as compared with logarithmically growing cells.

Increased IRES-mediated translation during differentiation requires eIF2α phosphorylation

The phenomenon of increased IRES-mediated translation under conditions of increased eIF2α phosphorylation, raised the notion that eIF2α phosphorylation confers a translational advantage on IRES-containing mRNAs. To test this hypothesis, we looked at the effect of expression of eIF2α phosphorylation inhibitors on IRES use. We used either the vaccinia virus K3L gene that encodes an eIF2α homologue and pseudo-substrate inhibitor of eIF2α protein.

Table 1. Effect of differentiation on IRES activity. Each of the bicistronic plasmids harbouring the IRES elements indicated in Fig. 1 was transfected into K562 cells followed by further incubation under nondifferentiation or differentiation conditions and subsequent analysis of Renilla (R) and Firefly (F) luciferase activity. Each value represents the mean ± SE of three independent experiments. The fold induction values represent the F/R ratio in differentiated cells relative to the F/R ratio in nondifferentiated cells.

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<th>Non-differentiated cells</th>
<th>Differentiated cells</th>
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<tr>
<td></td>
<td>Renilla (U per 10^6 cells)</td>
<td>Firefly (U per 10^6 cells)</td>
</tr>
<tr>
<td>pLPL</td>
<td>59 ± 3.5</td>
<td>7.5 ± 0.6</td>
</tr>
<tr>
<td>pLML</td>
<td>94 ± 10</td>
<td>6.4 ± 0.07</td>
</tr>
<tr>
<td>pLVL</td>
<td>89 ± 10</td>
<td>42 ± 3.3</td>
</tr>
<tr>
<td>pLEL</td>
<td>78 ± 9</td>
<td>3.4 ± 0.4</td>
</tr>
<tr>
<td>pLL</td>
<td>52 ± 7</td>
<td>0.6 ± 0.08</td>
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kinases [18,26], or PKRΔ6, a dominant-negative variant of the dsRNA activated eIF2α-kinase, PKR [19]. To confirm the connection between eIF2α phosphorylation during differentiation and IRES activation, we also used a plasmid encoding a variant form of eIF2α in which Ser51 is replaced by an alanine residue (eIF2α-S51A). As this variant protein cannot undergo phosphorylation, it serves as a competitor that reduces the translational inhibitory effect of phosphorylated endogenous wild-type eIF2α [20]. Plasmids expressing K3L, PKRΔ6, eIF2α-S51A variant or GFP as control, were cotransfected along with the different IRES-containing bi-cistronic vectors into K562 cells followed by their incubation under normal or differentiation conditions for 48 h prior to measurements of Renilla and firefly luciferase enzymatic activities. Tables 2 and 3 show the effects of the transfected gene products on the absolute levels of the translation products of both cistrons. Overexpression of K3L, PKRΔ6 or eIF2α-S51A led to enhanced translation of both cistrons in nondifferentiated cells, whereas in differentiated cells it led to decreased IRES-mediated translation of the second cistron. Fig. 4A summarizes the sensitivity of the differentiation-induced IRES activation to the various eIF2α phosphorylation inhibitors. Expression of eIF2α-S51A, K3L, or PKRΔ6 in differentiated cells reduced the level of eIF2α-P to 80%, 70% or 40% compared with GFP-transfected control, respectively (Fig. 4B). The effect of the various transfections on eIF2α phosphorylation is underestimated as not all the cells were successfully transfected. The reduction in IRES use in differentiated cells by expression of K3L, eIF2α-S51A and PKRΔ6 was shown to be correlated with a reduction in the level of eIF2α-P. These data suggest that eIF2α phosphorylation is required for more efficient IRES use during the differentiation process.
的那种抑制 eIF2α phosphorylation on IRES activity. The transfection efficiency was 50–60%, as judged by the percentage of fluorescent GFP-expressing cells. As shown in Fig. 4A, the differentiation process was not significantly affected by any of the transfected plasmids, as judged by the decreased number of cells in S-phase and increased number of polyploid cells. For additional confirmation we checked the level of the Vav proto-oncogene, which is known to increase early during megakaryocytic differentiation [27,29]. CKIIα protein level was used as a control. As shown in Fig. 5B, the level of Vav protein was increased due to reduced eIF2α phosphorylation level on the differentiation process. These results suggest that interference with eIF2α phosphorylation does not prevent the early differentiation steps, e.g. the global changes in gene expression upstream of mRNA translation. Instead, it interferes with the ability to fine-tune the translation efficiency of specific mRNA groups.

**DISCUSSION**

Cells undergoing terminal differentiation exhibit extensive changes in the pattern of gene expression. Much data has been accumulated regarding transcriptional regulation, but less is known about the mechanisms that inhibit the translation of most transcripts while activating the translation of specific mRNAs during the course of differentiation. During the early developmental stages of *Xenopus, Caenorhabditis elegans* and *Drosophila*, the translation of subclasses of mRNAs is regulated. However, during differentiation of mammalian cells, only a few individual mRNAs are known to be subjected to translational regulation due to their cis-regulatory elements (reviewed in [30]). Initial attempts to identify groups of translationally regulated genes during HL60 cell differentiation towards monocytes/macrophages has revealed that while most mRNAs are released from polysomes early in the differentiation process, a subset of transcripts is retained or even mobilized onto polysomes [7]. The data presented in this study suggest that mRNAs harbouring an IRES within long, structured, uORF-unburdened 5′UTRs, comprise a subgroup which is specifically translationally activated during differentiation (Fig. 1

### Table 2. Effect of K3L and PKRΔ6 expression on IRES activity. Absolute values of Renilla and Firefly activities from experiments described in Fig. 4A.

<table>
<thead>
<tr>
<th>Non-differentiated cells</th>
<th>Differentiated cells</th>
<th>Fold F/R induction</th>
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<tbody>
<tr>
<td></td>
<td>Renilla (U per 10^6 cells)</td>
<td>Firefly (U per 10^6 cells)</td>
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<td></td>
<td></td>
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<tr>
<td>pLPL + GFP</td>
<td>53 ± 3</td>
<td>6.4 ± 0.6</td>
</tr>
<tr>
<td>+ K3L</td>
<td>184 ± 13</td>
<td>20 ± 2</td>
</tr>
<tr>
<td>+ PKRΔ6</td>
<td>132 ± 12</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>pLML + GFP</td>
<td>103 ± 12</td>
<td>7.3 ± 1.1</td>
</tr>
<tr>
<td>+ K3L</td>
<td>303 ± 27</td>
<td>20 ± 3.5</td>
</tr>
<tr>
<td>+ PKRΔ6</td>
<td>222 ± 25</td>
<td>22 ± 3</td>
</tr>
<tr>
<td>pLVL + GFP</td>
<td>100 ± 11</td>
<td>52 ± 7</td>
</tr>
<tr>
<td>+ K3L</td>
<td>260 ± 21</td>
<td>130 ± 10</td>
</tr>
<tr>
<td>+ PKRΔ6</td>
<td>186 ± 20</td>
<td>120 ± 15</td>
</tr>
<tr>
<td>pLML + GFP</td>
<td>51 ± 6</td>
<td>2.0 ± 0.9</td>
</tr>
<tr>
<td>+ K3L</td>
<td>228 ± 24</td>
<td>8.5 ± 0.3</td>
</tr>
<tr>
<td>+ PKRΔ6</td>
<td>98 ± 10</td>
<td>5.7 ± 0.5</td>
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### Table 3. Effect of eIF2α-S51A expression on IRES activity. Absolute values of Renilla and Firefly activities from experiments described in Fig. 4A.

<table>
<thead>
<tr>
<th>Non-differentiated cells</th>
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<tbody>
<tr>
<td></td>
<td>Renilla (U per 10^6 cells)</td>
<td>Firefly (U per 10^6 cells)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pLPL + GFP</td>
<td>87 ± 6</td>
<td>7.8 ± 1.4</td>
</tr>
<tr>
<td>+ eIF2α-S51A</td>
<td>145 ± 11</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>pLVL + GFP</td>
<td>105 ± 11</td>
<td>42 ± 5</td>
</tr>
<tr>
<td>+ eIF2α-S51A</td>
<td>180 ± 20</td>
<td>72 ± 8</td>
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levels of active IRES elements have been found to confer a translational advantage under reduced global protein synthesis, such as reduced phosphorylation of eIF2α mediated by cellular IRES elements benefits from phosphorylation inhibitors on the differentiation process. Thus, the over-expression of the eIF2α phosphorylation inhibitor phosphor-mutant (eIF2α-S51A), or the pseudosubstrate K3L, resulted in reduced IRES activity in differentiated cells. However, it did not have any inhibitory effect on IRES activity in non-differentiated cells. It seems likely that the regulatory function of eIF2α depends on the delicate balance of phosphorylated eIF2α with other cellular components, and on the physiological status of the cell. The data presented in this study support this idea. Inhibition of PKR activity by over-expression of its dominant-negative variant PKRAΔ6, or reduction of eIF2α phosphorylation level by over-expression of its variant form, eIF2α-S51A, or the pseudosubstrate K3L, resulted in reduced IRES activity in differentiated cells. However, it did not have any inhibitory effect on IRES activity in non-differentiated cells (Fig. 4). The fact that expression of PKRAΔ6 had a greater impact on the levels of eIF2α phosphorylation and IRES-mediated translation compared with the efficient general eIF2α kinase inhibitor K3L suggests that PKR is the primary activated kinase during differentiation. Interestingly, the over-expression of the eIF2α phosphorylation inhibitors on IRES activity. (A) Each of the bicistronic vectors pLPL, pLML, pLVL, and pLEL (described in Fig. 1A) harbouring the IRES elements of PDGF2, VEGF, c-Myc or EMCV, respectively, was cotransfected into K562 cells together with a plasmid expressing the PKRAΔ6, K3L, eIF2α Ser51 → Ala mutant (p2α-S51A), or GFP coding region from the CMV promoter. The cells were further incubated under normal or differentiation conditions for 48 h and subsequently analysed for Renilla (R) and firefly (F) luciferase activity. (B) Fifty µg of total protein extract from differentiated cells transfected with plasmids expressing GFP, eIF2α-S51A, K3L or PKRAΔ6, as indicated. The transfected cells were incubated under normal (+TPA) or differentiation (+ TPA) conditions for 48 h, and subjected to DNA content analysis by flow cytometry. (B) One-hundred and forty µg of total protein extracted from the transfected cells as detailed in (A) were separated by SDS/10% PAGE, blotted onto a nitrocellulose membrane, and subjected to Western analysis using antibodies specific for Vav and for CKIIα.

Fig. 4. Effect of eIF2α phosphorylation inhibitors on IRES activity. (A) Each of the bicistronic vectors pLPL, pLVL, pLML, pLEL (described in Fig. 1A) harbouring the IRES elements of PDGF2, VEGF, c-Myc or EMCV, respectively, was cotransfected into K562 cells together with a plasmid expressing the PKRAΔ6, K3L, eIF2α Ser51 → Ala mutant (p2α-S51A), or GFP coding region from the CMV promoter. The cells were further incubated under normal or differentiation conditions for 48 h and subsequently analysed for Renilla (R) and firefly (F) luciferase activity. The absolute values are presented in Tables 2 and 3. Each value represents the mean ± SE of three independent experiments. The fold induction values represent the F/R ratio in differentiated cells relative to the F/R ratio in nondifferentiated cells. The graph demonstrates the effect of K3L (stippled bars), PKRAΔ6 (dark bars), or eIF2α-S51A (hatched bars) on the differentiation-induced IRES activation relative to the fold induction value with GFP that was set as 100% (light bars). (B) Fifty µg of total protein extract from differentiated cells transfected with plasmids expressing GFP, K3L, PKRAΔ6 or eIF2α-S51A were separated by 10% SDS/PAGE and blotted onto a nitrocellulose membrane. Phosphorylated eIF2α was detected using antibodies specific for phosphorylated Ser51. The same membrane was stripped and used for Western analysis using antibodies specific for total eIF2α. The eIF2α-P/eIF2α ratio in GFP-transfected cells was set as 1.
inhibitors did not interfere with the global process of megakaryocytic differentiation as judged by their morphology (not shown), cell-cycle, and enhanced Vav protein expression (Fig. 5). The latter result suggests that both eIF2α phosphorylation and IRES activation are late events during the differentiation process. The peak of PDGF2 IRES activation at 48 h after induction of differentiation [11,12] is in agreement with this notion. It is therefore conceivable that eIF2α phosphorylation serves to fine-tune the translation efficiency of specific mRNA groups.

Increased translation of certain IRES-containing mRNAs has also been implicated in apoptosis [40–43], a cellular process that includes activation of eIF2α phosphorylation [44–47]. Furthermore, the recently discovered cell cycle-dependent IRES elements are activated specifically at the G2/M boundary [48–50], when increased phosphorylation of eIF2α is found in correlation with decreased overall rate of protein synthesis [51]. Moreover, the IRES elements of the amino acid transporter protein cat-1 and c-Myc mRNAs have recently been shown to function efficiently where there is an increase in eIF2α phosphorylation, under conditions of amino acid starvation and genotoxic stress, respectively [52,53].

What is the mechanism underlying IRES-mediated translation under conditions of enhanced eIF2α phosphorylation? In nondifferentiated cells, in which global translation is active, the IRES-containing mRNAs compete with the cap-dependent mRNAs for the translation machinery. The decrease in global protein synthesis and reduced competition might be beneficial for IRES-mediated translation during differentiation. An interesting possibility may be the ability of IRES elements to direct efficient translation initiation in the absence of eIF2 and Met-tRNA. Recently, internal initiation without Met-tRNA has been demonstrated in two picorna-like insect viruses, Plantia stall intestinal virus and Cricket paralysis virus [54–56]. It is an open question whether cellular IRES elements known to contain conserved secondary and tertiary structural motifs can also direct internal translation from noncognate initiation codons in the absence of Met-tRNA. Direct binding to the 40 S ribosomal subunit followed by joining of the 60 S subunit may provide a significant advantage to IRES elements that confer efficient translation under conditions of global translation inhibition mediated by eIF2α phosphorylation. Another possibility could be that a mechanism exists that is similar to the translational regulation of GCN4 in yeast. In this case, the induction of GCN4 translation in response to eIF2α phosphorylation is modulated by four short uORFs in the 5′UTR. Reduced rates of ternary complex formation leads to bypass of the uORFs and initiation at the downstream GCN4 major ORF [6]. A comparable mechanism of translational regulation was recently demonstrated for the stress-induced transcription factor ATF4, in mammalian cells under conditions of enhanced eIF2α phosphorylation due to stress [57]. Similarly, the short ORFs that furnish many of the cellular IRES elements may have a role in translational regulation, as seems to be the case for the activation of the cat-1 mRNA [52]. Internal ribosomal binding upstream of the translation initiator codon may be followed by sequential scanning to the initiation codon. For instance, in the case of the PDGF2 5′UTR, which contains three uORFs, the IRES has been mapped to the central part of the 5′UTR at the vicinity of the first uORF [13]. The need to scan through the second and third uORFs towards the major coding region cannot be ruled out at this point. Another possibility may be that eIF2α phosphorylation induces the synthesis of a protein that interacts with the IRES. This can be achieved by direct translational regulation of its mRNA akin to GCN4/ATF4 mRNAs, or by regulating the translation of a GCN4/ATF4-like transcription factor that activates the transcription of the potential IRES activator. Current experiments are designed to elucidate the mechanism(s) by which eIF2α phosphorylation serves to enhance IRES-mediated translation.

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