IRES-dependent translation of *egr2* is induced under inflammatory conditions

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ABSTRACT

Adjusting translation is crucial for cells to rapidly adapt to changing conditions. While pro-proliferative signaling via the PI3K-mTOR-pathway is known to induce cap-dependent translation, stress conditions, such as nutrient deprivation or hypoxia often activate alternative modes of translation, e.g., via internal ribosome entry sites (IRESs). As the effects of inflammatory conditions on translation are only poorly characterized, we aimed at identifying translationally deregulated targets in inflammatory settings. For this purpose, we cocultured breast tumor cells with conditioned medium of activated monocyte-derived macrophages (CM). Polysome profiling and microarray analysis identified early growth response-2 (*egr2*) to be regulated at the level of translation. Using bicistronic reporter assays, we found that *egr2* contains an IRES within its 5' UTR, which facilitated enhanced translation upon CM treatment. We further provide evidence that the activity of *egr2*-IRES was induced by IL-1 β and p38-MAPK signaling. In addition, we identified several potential IRES *trans*-acting factors (ITAFs) such as polypyrimidine tract binding protein (PTB) and hnRNP-A1 that directly bind to the *egr2*-5'UTR. In summary, our data provide evidence that *egr2* expression is translationally regulated via an IRES element, which is responsive to an inflammatory environment.

Keywords: *egr2*; translation; IRES; inflammation; IL-1 β ; p38-MAPK

INTRODUCTION

Regulation of translation primarily takes place at the level of translation initiation. This process involves the assembly of the translation initiation complex at the 5' untranslated region (5' UTR) of the mRNA to recruit the ribosomes (Sonnenberg and Hinnebusch 2009; Silvera et al. 2010). Formation of the initiation complex, comprising eukaryotic initiation factors (eIFs) such as the RNA helicase eIF4A, the scaffolding protein eIF4G, and the cap-binding protein eIF4E, is highly regulated by the PI3K-mTOR pathway (Guertin and Sabatini 2007). Specifically, mTOR phosphorylates and thereby activates p70^{S6K}, which in turn phosphorylates the 40S ribosomal subunit, a critical step in translation initiation. Moreover, mTOR inhibits the 4E-binding protein (4E-BP), which, in a hypophosphorylated state, sequesters

and, thus, inhibits the cap-binding protein eIF4E. Hyperphosphorylation of 4E-BP by mTOR releases eIF4E, which then interacts with the cap-structure of mRNAs to recruit other eIFs as well as the ribosomal subunits (Pause et al. 1994).

Enhanced activation of PI3K-mTOR signaling has been shown to specifically stimulate the translation of various tumor-associated factors with highly structured 5' UTRs such as cyclin D1 (Averous et al. 2008). Thus, deregulated translation is increasingly being used as a target for the development of novel tumor therapeutics. So far translation-oriented therapies predominantly target the kinase mTOR (e.g., rapamycin and its analogs) (Faivre et al. 2006). Yet, under conditions where cap-dependent translation is inhibited, the protein synthesis of various survival genes is maintained in a cap-independent manner, e.g., via internal ribosome entry sites (IRESs) (Holcik 2004). IRES elements facilitate initiation of translation independently of the cap-binding protein eIF4E. Such elements were described for various oncogenes like Hif-1 α (Lang et al. 2002) and Bcl-2 (Sherrill et al. 2004). In most cases, activation of IRES elements is mediated via IRES *trans*-acting factors (ITAFs),

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such as the polypyrimidine tract binding protein (PTB), which bind to short sequences or secondary structures of the 5' UTR (Spriggs et al. 2005). Binding of these proteins initiates conformational changes of the 5' UTR structure, thereby allowing the interaction of eIFs and ribosomal subunits with the mRNA to facilitate translation.

While translation initiation is modulated by a variety of stimuli including mitogens (Sonenberg and Hinnebusch 2007), hormones (Proud 2006), nutrients (Proud 2004), and stress signals (Wek et al. 2006), little is known about the effect of inflammation on translational regulation. Therefore, the present study aimed at identifying translationally deregulated targets during inflammation-associated tumorigenesis. Using polysome profiling and microarray analysis of breast tumor cells treated with supernatants of activated macrophages, we identified genes that were regulated predominantly at the level of translation in the tumor cells. Among these, early growth response-2 (*egr2*) was identified as a novel translationally regulated target. Translation of *egr2* was induced by IL-1 β and subsequent p38-mitogen-activated protein kinase (MAPK) activation. In addition, we identified an IRES element within the 5' UTR of *egr2* and found several potential ITAFs such as PTB and hnRNP-A1 to bind to the 5' UTR of *egr2*. Thus, we propose that *egr2* translation is regulated in an IRES-dependent manner by IL-1 β and p38-MAPK.

RESULTS

Inflammatory conditions provokes translational changes of specific mRNAs

In order to identify proteins that are translationally regulated under inflammatory conditions, we established an in vitro model for macrophage-associated inflammation. Briefly, we used supernatants of activated U937 monocyte-derived macrophages (CM) which we have previously shown to contain significantly elevated protein levels of the proinflammatory mediators TNF α , IL-6, and IL-8 as compared to supernatants of undifferentiated U937 monocytes (Ctr) (Schmid et al. 2011). Additionally, CM enhanced AP1-transactivation and degradation of the tumor suppressor Pdc4 (Yasuda et al. 2010), thereby providing evidence for the induction of a protumorigenic program in MCF7 cells.

To search for proteins that are regulated at the translational level, MCF7 cells were treated for 4 h with CM or Ctr, followed by polysomal fractionation. To this end, cytoplasmic lysates were generated and layered on a sucrose gradient leading to sedimentation of mRNAs according to their ribosome occupancy after ultracentrifugation (Fig. 1). Notably, the polysome profile of MCF7 cells treated with CM did not show obvious differences compared to Ctr (Supplemental Fig. S1A,B). The ribosome distribution was confirmed in single fractions using a denaturing agarose

FIGURE 1. Experimental setup. U937 cells were differentiated with 10 nM TPA for 48 h or left untreated. Then, cells were trypsinized, washed with PBS, and reseeded in fresh medium. After 24 h, the conditioned medium from activated U937 monocyte-derived macrophages (CM) or undifferentiated U937 monocytes (Ctr) was harvested. MCF7 cells were incubated for 4 h with CM or Ctr. Subsequently, the respective cellular lysates were subjected to polysomal fractionation. Equal aliquots of RNA isolated from single fractions were analyzed using denaturing agarose gel electrophoresis to verify 28S and 18S rRNA distribution as indicators for ribosome distribution. Pooled polysomal fractions 6–10 and total RNA were analyzed using whole genome microarrays. Comparing polysomal changes (CM vs. Ctr) with total changes (CM vs. Ctr) revealed translationally regulated genes.

gel to visualize 28S and 18S rRNA (Fig. 1). For global analysis of translationally regulated mRNAs, polysomal fractions 6–10 were pooled and subjected to microarray analysis. In parallel, microarray analysis of total mRNA was performed. mRNAs that significantly changed in the polysome-associated portion but not on the total level were considered as translationally regulated. Among the mRNAs identified to shift into or out of the polysomal fractions, *egr2*, *plaur*, *map3k5*, *gfra2*, and *cblb* showed the strongest translational induction (2.5- to 7.0-fold), whereas *apq11*, *fgd3*, *rnf214*, *fance*, and *lrrc20* displayed the strongest translational repression (–2.2- to –2.7-fold) (Table 1).

Egr2 is translationally up-regulated under inflammatory conditions

As early growth response-2 showed the highest increase in polysomal RNA of all targets (7.0-fold), we chose this target for further investigation. To this end, we analyzed the relative amount of *egr2* mRNA in single fractions after Ctr or CM treatment and found that *egr2* mRNA distribution decreased in the monosomal and increased in the polysomal fraction in response to CM (Fig. 2A). In contrast, the mRNA distribution of *gapdh* was not affected by CM (Fig. 2B). To

TABLE 1. List of highest translationally up- and down-regulated genes

Symbol	Poly change	P-value	Acc. no.
egr2	7.0	1.99×10^{-04}	NM_000399.2
plaur	3.9	7.03×10^{-04}	NM_002659.2
map3k5	2.9	4.24×10^{-04}	NM_005923.3
gfra2	2.6	2.29×10^{-03}	NM_001495.4
cblb	2.5	1.77×10^{-03}	NM_170662.3
aqp11	-2.7	1.17×10^{-02}	NM_173039.1
fgd3	-2.5	2.16×10^{-02}	NM_033086.2
rnf214	-2.4	3.58×10^{-03}	NM_207343.2
fance	-2.2	7.41×10^{-03}	NM_021922.2
lrrc20	-2.2	2.70×10^{-03}	NM_018239.2

allow for statistical evaluation of the observed changes, mRNA distribution in response to CM was normalized to the Ctr distribution for individual experiments. As predicted based on the individual experiments, *egr2* mRNA distribution significantly decreased in monosomal fractions 1–3, while it significantly increased in polysomal fractions 6–10 (Fig. 2C). Again, no difference was observed for *gapdh* (Fig. 2D). For control purposes, we determined changes in the mRNA distribution of the related *egr3*, which was not identified to be translationally regulated in the microarray analysis. As expected, no significant changes could be observed for the *egr3* mRNA distribution in response to CM (Fig. 2E). To get a measure for overall CM-induced translational changes, the mRNA abundance in pooled polysomal fractions 6–10 was determined relative to Ctr and yielded a significant increase of 1.96 ± 0.13 for *egr2*, whereas *egr3* remained unaltered (Fig. 2F).

These data indicate that *egr2* translation is specifically induced under inflammatory conditions.

IL-1 β induces translational up-regulation of *egr2*

To elucidate molecular mechanisms and pathways enhancing translation of *egr2*, we initially analyzed changes of mRNA expression under these conditions. One hundred fifty-seven genes were found to change more than twofold on total mRNA level. These targets were subjected to pathway analysis using Ingenuity software. Within the associated network functions, inflammatory and cancer responses were enriched. Similarly,

molecular functions were indicative of tumorigenic changes (cell growth, proliferation, and cellular movement) (Table 2). These results further support the concept that TPA-activated U937 monocyte-derived macrophages induce a protumorigenic and inflammatory response in MCF7 cells. We also found that these macrophages secrete physiological amounts of IL-1 β (91.7 ± 18.2 pg/mL), whereas no IL-1 β was secreted by undifferentiated U937 monocytes (Fig. 3A). We next asked whether IL-1 β might affect *egr2* translation in our system. To this end, we depleted IL-1 β in CM by preincubating CM with IL-1 β neutralizing antibody for 1 h. As compared to the IgG control-treated CM, *egr2* mRNA in the pooled polysomal fractions 6–10 was significantly reduced to 0.86 ± 0.03 in response to IL-1 β -depleted CM (Fig. 3B). To determine if IL-1 β alone is able to induce *egr2* translation, we treated MCF7 cells with recombinant IL-1 β (50 ng/mL) for 4 h and observed a significantly increased amount of *egr2* mRNA in the pooled polysomal fractions (1.64 ± 0.11 relative to the control) (Fig. 3C).

Taken together, these data indicate that IL-1 β suffices to induce *egr2* translation. Moreover, IL-1 β appears to contribute to the induction of *egr2* translation in response to conditioned medium from activated monocyte-derived macrophages.

FIGURE 2. *Egr2* is translationally up-regulated. MCF7 cells were treated with CM (black diamonds, solid line) or Ctr (white squares, dashed line) for 4 h and subjected to polysomal fractionation. RNA was isolated from single fractions and analyzed using RT-qPCR. The relative mRNA distribution of *egr2* (A) and *gapdh* (B) is shown. Graphs are representative for at least three independent experiments. The ratio of mRNA distribution of CM to Ctr was calculated to visualize stimulus-dependent changes in single fractions for *egr2* (C), *gapdh* (D), and *egr3* (E). (F) Pooled polysomal changes (fractions 6–10) of *egr2* (left panel) and *egr3* (right panel) in response to CM were determined relative to Ctr and normalized to *gapdh*. Data are presented as means $\pm$ SEM ($n > 3$). (***) $P < 0.001$.

TABLE 2. IPA of total changes

Associated network functions	Molecules
1. Cell death, proliferation, inflammatory response	39
2. Inflammatory response and disease	32
3. Cancer, cell cycle	17
Molecular and cellular functions	
1. Cell death	53
2. Cellular growth and proliferation	56
3. Cellular movement	34

IL-1 β -induced translational activation of *egr2* is p38-MAPK-dependent

We next aimed at understanding the signaling cascades linking *egr2* translation and IL-1 β . IL-1 β is known to induce p38-MAPK activity via phosphorylation (Ichijo 1999). Accordingly, we found that p38-MAPK is rapidly and transiently phosphorylated in MCF7 cells in response to IL-1 β treatment (Fig. 4A). Interestingly, a similar time course of p38-MAPK-phosphorylation was observed upon treatment with CM, whereas Ctr did not affect phosphorylation, i.e., activation of p38-MAPK (Fig. 4B). We then examined whether this pathway is involved in enhanced translation of *egr2* under these conditions. Therefore, we cotreated MCF7 cells with CM in combination with the p38-MAPK inhibitor SB203580 (10 μ M). Inhibition of p38-MAPK did not affect global translation (Supplemental Fig. S1) but specifically decreased *egr2* mRNA in the pooled polysomal fractions 6–10 to 0.84 ± 0.01 relative to CM-treated cells (Fig. 4C; Supplemental Fig. S2). To verify that IL-1 β contributed to the activation of p38-MAPK in response to CM, we employed CM depleted for IL-1 β by a neutralizing antibody approach. Indeed, neutralizing IL-1 β attenuated CM-induced phosphorylation of p38-MAPK (Fig. 4D).

In summary, these results indicate that IL-1 β increases *egr2* translation in a p38-MAPK-dependent manner.

Various ITAFs bind to the 5' UTR of *egr2*

As translation of individual mRNAs is often regulated via their 5' UTR (Pickering and Willis 2005), we next analyzed the 5' UTR of *egr2*. The 5' UTR of *egr2* proved to be relatively long, being composed of 326 bases (Fig. 5A). To determine potential structures in silico, we used *mfold* (Zuker 2003), which predicted a secondary structure containing various loops with a minimal free energy of -89.40 kcal/mol (Fig. 5B). As complex secondary structures commonly require RNA-binding proteins for efficient translation, we performed streptavidin-tethered RNA-affinity purification with the 5' UTR of *egr2*. For this purpose, we transcribed the 5' UTR of *egr2* and conjugated a biotin-label at the 5'

end. The labeled transcript was then incubated with streptavidin agarose beads and protein lysate of 4-h CM-treated MCF7 cells. After elution and electrophoretic separation, proteins bound to the 5' UTR of *egr2* were analyzed by mass spectrometry. A number of heterogeneous ribonucleoproteins (hnRNPs) and eukaryotic initiation factors were identified to bind to the 5' UTR of *egr2*, which did not precipitate in the control reaction using non-biotinylated RNA (Fig. 5C). Among the *egr2*-5'UTR binding proteins, we found PTB, hnRNP-A1, and human antigen R (HuR), all of which were previously shown to act as ITAFs associated with enhanced translation of target mRNAs (Vagner et al. 2001; Bonnal et al. 2005; Cobbold et al. 2010). To ensure an RNA-specific binding of these proteins to the *egr2*-5'UTR, the interaction was further verified via RNA-affinity purification, followed by Western analysis, including a control RNA of similar length (300 nt) encoding a fraction of

FIGURE 3. IL-1 β induces translational up-regulation of *egr2*. (A) Secretion of IL-1 β in Ctr and CM was analyzed using CBA assays ($n > 3$). (**) $P < 0.01$. (B) CM was pretreated with IgG control (5 μ g/mL) or neutralizing IL-1 β antibody (5 μ g/mL) for 1 h at 37°C. Then, MCF7 cells were incubated with the respective CMs for 4 h, followed by polysomal fractionation as described before. Changes of *egr2* mRNA in pooled polysomal fractions 6–10 in response to CM+ α IL-1 β relative to CM+IgG were determined. (C) MCF7 cells were treated with human recombinant IL-1 β (50 ng/mL) for 4 h, followed by polysomal fractionation as described before. IL-1 β -induced changes for *egr2* mRNA in pooled polysomal distribution relative to control were analyzed using RT-qPCR. Data are normalized to *gapdh* and presented as means $\pm$ SEM ($n \geq 3$). (*) $P < 0.05$.

FIGURE 4. CM induces *egr2* translation in a p38-MAPK-dependent manner. (A) MCF7 cells were treated with human recombinant IL-1 β (50 ng/mL) to the indicated time points. Whole-cell extracts were subjected to Western analysis and probed with the indicated antibodies. Blots are representative for at least three independent experiments. (B) MCF7 cells were treated with CM or Ctr to the indicated time points. Whole-cell extracts were subjected to Western analysis and probed with the indicated antibodies. Blots are representative for at least three independent experiments. (C) MCF7 cells were treated for 4 h with CM alone or in combination with SB203580 (10 μ M), followed by polysomal fractionation. Change of *egr2* mRNA in pooled polysomal fractions 6–10 in response to CM+SB is given relative to CM. Data are normalized to *gapdh* and presented as means $\pm$ SEM ($n \geq 3$). (**) $P < 0.01$. (D) MCF7 cells were treated for 15 min with Ctr and CM pretreated with IgG (5 μ g/mL) or IL-1 β neutralizing antibody (5 μ g/mL) as described before. Whole-cell extracts were subjected to Western analysis and probed with the indicated antibodies, followed by densitometric analysis. Levels of phospho-p38 were normalized to total p38 and are presented relative to Ctr. Blots are representative for at least three independent experiments.

human *gapdh* in reverse orientation (*hrg*). While PTB, hnRNP-A1, and HuR bound to *egr2*-5'UTR, no relevant binding to the control was observed (Fig. 5D). The minimal pulldown of HuR detected with the control appeared negligible compared to the amount of HuR pulled down with the *egr2*-5'UTR. Thus, our data support a specific binding of these three potential ITAFs to the *egr2*-5'UTR. However, we could not observe a differential binding when comparing CM- and Ctr-treated MCF7 lysates (data not shown).

Since binding of potential ITAFs indicates that an IRES element could be located in the 5' UTR of *egr2*, we next tested whether inhibition of cap-dependent translation, in general, has an impact on *egr2* translation. For this purpose, we treated MCF7 cells with the mTOR inhibitor rapamycin to inhibit cap-dependent translation and followed the polysomal distribution changes of mRNA in single fractions. As expected, *gapdh* mRNA abundance increased in monosomal fractions and decreased in polysomal fractions, indicative of an mRNA being translated cap-dependently

(Fig. 5E). Interestingly, *egr2* translation appeared to be enhanced under these conditions, supporting cap-independent translation of *egr2*.

Taken together, the long, structured 5' UTR, the binding of potential ITAFs, and enhanced translation under conditions of inhibited cap-dependent translation were taken as indicators that *egr2* mRNA is translated in an IRES-dependent manner.

Egr2-5'UTR contains an IRES element

To determine the presence of IRES elements within the 5' UTR of *egr2*, we used a bicistronic reporter plasmid. This construct expresses *renilla* luciferase in a cap-dependent manner, whereas the *firefly* luciferase is only translated when a functional IRES element is present. In addition, a hairpin sequence inserted upstream of the *renilla* luciferase sequence served to minimize cap-dependent translation and read-through across the *renilla* termination codon (Fig. 6A). To determine the presence of an IRES within the 5' UTR of *egr2*, *egr2*-5'UTR was inserted into the intercistronic region of *phpRF*, resulting in *phpR-egr2-F*. Cells transiently cotransfected with *phpRF* or *phpR-egr2-F* in combination with the β -galactosidase transfection control showed similar relative *renilla* activities (Fig. 6B). In contrast, the relative *firefly* activity was significantly higher in the presence of *egr2*-5'UTR (7.12 ± 0.61 -fold compared to *phpRF*).

To gain final proof for the presence of an IRES within the *egr2*-5'UTR, we transfected in vitro transcribed mRNA of the bicistronic construct instead of DNA. While *renilla* luciferase signals remained at similar levels, *firefly* activity was again enhanced 12.3 ± 0.83 -fold, when comparing *hpR-egr2-F* mRNA to *hpRF* mRNA (Fig. 6C). This observation served as unequivocal proof for the presence of an IRES within the 5' UTR of *egr2*. To rule out the contribution of cryptic promoter activity to the activity of the bicistronic constructs transfected as DNA, we introduced the *egr2*-5'UTR into the promoterless pGL3-basic vector. Insertion of *egr2*-5'UTR did not enhance *firefly* activity in transiently transfected MCF7 cells compared to the pGL3-basic parent vector. In contrast, the positive control containing an SV40 promoter showed a strong relative luciferase activity compared to the promoterless vector (Fig. 6D). Thus, cryptic promoter activity was excluded. To assess if cryptic splicing might occur, we checked whether the bicistronic plasmid generates an mRNA transcript of the expected size. Specifically, the full-length transcript of the *phpR-egr2-F* (*R-egr2-F*) was predicted to contain 2933 nt, whereas the *phpRF* control vector should produce a transcript (*RF*) of 2587 nt. For this purpose, we treated mRNA isolated from cells transfected with either *phpRF* or *phpR-egr2-F* with DNase to remove residual contaminations of the plasmid DNA. PCR with primers that specifically bind to the 5' end of *renilla* and 3' end of *firefly* open reading frames

FIGURE 5. PTB binds to *egr2*-5'UTR. (A) Sequence of human *egr2*-5'UTR. (B) Predicted structure of the human *egr2*-5'UTR using *mfold*. (C) Lysates of CM-treated MCF7 cells were incubated with in vitro transcribed, biotinylated *egr2*-5'UTR. After immunoprecipitation using streptavidin agarose beads, bound proteins were separated on SDS-PAGE and visualized using silver staining. Individual proteins were identified by mass spectrometric analysis of gel slices. Positions of identified proteins are shown on the left side, protein molecular weight markers are shown on the right side. (D) Lysates of CM-treated MCF7 cells were incubated with in vitro transcribed, biotinylated *egr2*-5'UTR or human reverse *gapdh* (*hrg*) RNA, before immunoprecipitation of proteins using streptavidin agarose beads. Bound proteins were subjected to Western analysis and probed with the indicated antibodies. Blots are representative for at least three independent experiments. (E) MCF7 cells were treated with rapamycin (100 nM) for 4 h and subjected to polysomal fractionation. RNA from single fractions was isolated and *egr2* and *gapdh* mRNA distribution changes were analyzed using RT-qPCR. Data are presented as means $\pm$ SEM ($n = 3$).

to amplify the full-length RL or R-*egr2*-L mRNAs resulted in a single product of the expected size for each vector (Fig. 6E). While this observation verifies the generation of the full bicistronic mRNA, it does not rule out the presence of additional monocistronic *firefly* mRNAs. Therefore, we determined the expression of both *renilla* and *firefly* mRNAs in cells transfected with either phpRF control or phpR-*egr2*-F vectors. The ratio of *firefly* to *renilla* mRNA was not altered upon insertion of the *egr2*-5'UTR compared to the empty vector control (Fig. 6F); thus, cryptic splicing and cryptic promoter activity were excluded.

Taken together, these experiments indicate that cryptic promoter and splice sites do not account for the observed *egr2*-5'UTR-dependent increase in the bicistronic *firefly* signal. Furthermore, they strongly support the presence of an IRES element in the 5' UTR of *egr2*.

We next determined if the IRES activity, defined as the ratio of *firefly* to *renilla* luciferase activities, responds to CM. As predicted, IRES activity of phpR-*egr2*-F transfected cells was significantly induced in response to 24 h CM (2.30 ± 0.22 -fold compared to Ctr) (Fig. 6G).

In the last set of experiments, we aimed at determining if the CM-induced IRES activity was also dependent on IL-1 β and p38-MAPK activation. To this end, cells were transfected with phpR-*egr2*-F. Treatment with CM for 4 h led to a significant 1.5 ± 0.18 -fold increase in *egr2*-IRES activity. Similarly, treatment with recombinant IL-1 β alone resulted in a significant 1.34 ± 0.11 -fold *egr2*-IRES activation compared to Ctr cells (Fig. 7A). Importantly, CM-induced *egr2*-IRES activity was significantly reduced by the p38-MAPK inhibitor SB203580 to $82 \pm 4\%$ (Fig. 7B).

Thus, we conclude that within an inflammatory environment, IL-1 β and p38-MAPK contribute to *egr2*-IRES-dependent translation.

DISCUSSION

In this report, we identify *egr2* as a novel translationally regulated target. Specifically, we show that *egr2* mRNA shifts from monosomal to polysomal fractions upon treatment with supernatants of activated monocyte-derived macrophages that were previously shown to induce a proinflammatory as well as a protumorigenic program in MCF7 cells (Schmid et al. 2011). Enhanced *egr2* translation resulted from IL-1 β -dependent p38-MAPK-mediated activation of a newly identified IRES within the 5' UTR of *egr2*.

So far, regulation of *egr2* is only poorly understood. Some studies suggested transcriptional activation for *egr2* (Yokota et al. 2010; Fang et al. 2011). A recent report identified miR-17-92 as binding to the 3' UTR of *egr2*, reducing *egr2* levels either by mRNA destabilization or suppression of translation in murine macrophages during leukemogenesis (Pospisil et al. 2011). In our hands, inflammatory conditions did not affect translation of *egr2* via its 3' UTR as introducing the 3' UTR into a reporter vector left luciferase activities upon treatment with CM unaltered (data not shown). Importantly, transfection of in vitro transcribed mRNA of the bicistronic vectors validated the presence of an IRES within the 5' UTR of *egr2* (Fig. 6C). In addition, the contribution of cryptic promoters, splicing, or read-through was excluded (Fig. 6). Furthermore, we found that IL-1 β within the CM (Fig. 3A) contributed to enhanced IRES-dependent translation of *egr2* via p38-MAPK activation (Fig. 7A,B). While IL-1 β was previously shown to suppress translation of thrombomodulin (Yeh et al. 2008),

FIGURE 6. *Egr2* contains an IRES element. (A) Scheme of bicistronic constructs used for reporter assays. (B) Bicistronic reporter plasmids were cotransfected with SV40- β -Gal plasmid into MCF7 cells. Twenty-four hours after transfection, *renilla* and *firefly* activities were measured and normalized to β -galactosidase activity. Data are presented as means $\pm$ SEM ($n > 3$). (**) $P < 0.01$ relative to phpRF. (C) In vitro transcribed mRNAs of the indicated reporter plasmids were transfected into MCF7 cells. Twenty-four hours after transfection, *renilla* and *firefly* activities were measured. Data are presented as means $\pm$ SEM ($n > 3$). (**) $P < 0.01$ relative to hpRF. (D) MCF7 cells were cotransfected with the indicated reporter constructs and SV40-*renilla* plasmid. Forty-eight hours after transfection, *firefly* activity was measured and normalized to *renilla* activity. Data are presented as means $\pm$ SEM ($n > 3$). (E) RNA isolated from cells transfected with phpRF or phpR-egr2-F was DNase-treated. cDNA was synthesized, and PCR was performed with specific primers to amplify full-length RL or R-egr2-L mRNAs. PCR products were visualized via agarose gel electrophoresis. Data are representative for at least three independent experiments. (F) RT-qPCR analysis of the amount of *firefly* mRNA normalized to *renilla* mRNA. Data are presented as means $\pm$ SEM ($n = 3$). (G) MCF7 cells were transfected with phpR-egr2-F and treated with CM or Ctr for 24 h. IRES activity was calculated as the ratio of *firefly* to *renilla* activities and is given relative to Ctr. Data are presented as means $\pm$ SEM ($n > 3$). (**) $P < 0.01$.

no translation-inducing function of IL-1 β has been identified so far. Recently IL-1 α was reported to activate translation of mRNAs such as I κ B ζ and IL-6 (Dhamija et al. 2010). Moreover, IL-6 was shown to enhance IRES-dependent translation of prosurvival genes like c-myc (Shi et al. 2011). Notably, in our experiments, depletion of IL-1 β did not fully repress CM-induced *egr2* translation, and treatment with recombinant IL-1 β did not completely recapitulate enhanced *egr2* translation as seen in response to CM (Fig. 3B,C). Thus, besides IL-1 β , additional factors within the CM, which remain to be elucidated, contribute to enhanced *egr2* translation. Furthermore, our data corroborate previous reports showing that p38-MAPK activity is important for IRES-dependent translation of cyclin D1 and c-myc (Shi et al. 2005; Cloninger et al. 2011).

Further evidence for the presence of IRES elements within the 5' UTR of *egr2* comes from our finding that various proteins that were previously described to function as ITAFs for other mRNAs bind to this region (Fig. 5C). Especially, PTB is considered to activate IRESs of many IRES-bearing mRNAs (Sawicka et al. 2008; Yang et al. 2010). Interestingly,

among a number of hnRNPs, we also found hnRNP-A1, which was previously shown to be crucial for the IRES function of c-myc (Shi et al. 2008) and *fgf2* (Bonnal et al. 2005). While no differential binding of PTB and hnRNP-A1 was seen between CM- and Ctr-treated conditions, various other RNA-interacting proteins were found to bind to *egr2*-5'UTR as well. Interestingly, Papadopoulou et al. (2010) recently described a complex comprising a number of hnRNPs to interact with mRNAs. Changes in *egr2*-5'UTR binding complexes might, therefore, account for the translational regulation of *egr2*. As a detailed analysis of RNA-binding protein complexes and their subcellular localization is beyond the scope of the present study, future studies are required to identify those proteins that are critical for the regulation of IRES-dependent *egr2* translation. It can be speculated that either differential binding or recruitment of other factors is required to mediate the IRES-transactivating function under inflammatory conditions.

Functionally, *egr2* was described as a transcription factor belonging to the early growth response gene family that is activated immediately after serum addition (Joseph et al. 1988). It was shown to be crucial for hindbrain development (Nagarajan et al. 2001) and recently proposed to exert E3 SUMO ligase activity (Garcia-Gutierrez et al. 2011). Furthermore, *egr2* was identified as an important regulator of T-cell tolerance by inhibiting IL-2 production, promoting a T-cell receptor-induced negative regulatory program. *Egr2* was, therefore, proposed to be a good target in cancer therapy to evade tumor-induced immune tolerance (Safford et al. 2005). Its role in the regulation of cell proliferation is controversial, i.e., while *egr2* was shown to be induced in cells overexpressing tumor suppressor PTEN (Matsushima-Nishiu et al. 2001), it also appears to induce the prosurvival protein Mcl1 and to stimulate proteasomal degradation of the proapoptotic protein Bim (Bradley et al. 2008). Thus, while *egr2* translation is enhanced under conditions which we have previously shown to be protumorigenic (Yasuda et al. 2010; Schmid et al. 2011), the function of *egr2* in this setting remains to be elucidated.

Taken together, we provide evidence for a novel mechanism of *egr2* regulation via enhanced IRES-dependent translation under proinflammatory conditions. This effect is mediated by IL-1 β and other factors and involves

FIGURE 7. Egr2-IRES activity is p38-MAPK- and IL-1 β -dependent. (A) MCF7 cells were transfected with phpR-egr2-F. Forty-eight hours after transfection, cells were treated with Ctr, CM, or rIL-1 β (50 ng/mL) for 4 h. IRES activity was calculated as described before and is given relative to Ctr. Data are presented as means $\pm$ SEM ($n > 3$). (*) $P < 0.05$. (B) MCF7 cells were transfected with phpR-egr2-F. Forty-eight hours after transfection, cells were treated for 4 h with CM or CM containing SB203580 (10 μ M). IRES activity was calculated as described before and is given relative to CM. Data are presented as means $\pm$ SEM ($n > 3$). (**) $P < 0.01$.

p38-MAPK activation. As egr2 plays a crucial rule in T-cell tolerance, an in-depth understanding of the regulation of egr2 will be of interest for conditions where T-cell activation should be therapeutically altered, such as transplantations or tumor immunotherapies.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma-Aldrich, if not indicated otherwise. Antibodies were obtained from the following sources: anti-phospho-p38 (Thr180/Tyr182), anti-p38, and anti-hnRNP-A1 from Cell Signaling Technology, anti-nucleolin and anti-HuR from Santa Cruz Biotechnology, anti-IL-1 β and IgG isotype from R&D System GmbH, and IRDyes 680LT and 800CW secondary antibodies from Li-COR Biosciences GmbH. Anti-PTB was a kind gift of Prof. Anne Willis (Spriggs et al. 2009). Recombinant IL-1 β came from Peprotech. Rapamycin was purchased from Cell Signaling Technology and SB203580 from Enzo Life Science.

Cell culture

Cell lines came from LGC Standards GmbH. MCF7 and U937 cells were maintained in RPMI medium supplemented with 10% fetal

bovine serum (FBS), 100 U/mL penicillin, 100 μ g/mL streptomycin, and 2 mM L-glutamine. Additionally, U937 media contained 1 mM sodium pyruvate. Cells were kept at 37°C in a humidified atmosphere with 5% CO₂. Medium and supplements were purchased from PAA. FBS came from Biochrom.

Macrophage differentiation and conditioned medium

U937 monocytes (1×10^7 /25 mL) were exposed to TPA (10 nM) for 48 h. The resulting adherent, activated U937 monocyte-derived macrophages were trypsinized, pelleted, and washed with PBS. For control purposes, undifferentiated U937 monocytes (3×10^6 /25 mL) were incubated with DMSO (0.1%) for 48 h, pelleted by centrifugation, and washed with PBS. Subsequently, control and differentiated U937 were treated equally. For the generation of conditioned medium, U937 cells were reseeded at a concentration of 2×10^6 /5 mL. Cells were allowed to condition medium for 24 h followed by centrifugation, sterile filtration (0.45 μ m filter), and storage at -80°C until further use. All experiments were carried out in U937 medium. To determine the secretion of IL-1 β by the U937 cells, conditioned media were analyzed using the BD Cytometric Bead Array (CBA) Human Inflammation Kit according to the manufacturer's manual. Samples were measured using the BD LSRFortessa flow cytometer and analyzed with the BD Biosciences FCAP software (BD Biosciences).

Polysomal fractionation

7.5×10^6 MCF7 cells were seeded in a 15-cm dish 1 d prior to treatment of the cells, followed by polysomal fractionation. Briefly, after incubation with 100 μ g/mL cycloheximide (CHX) for 10 min at 37°C, cells were harvested in PBS/CHX (100 μ g/mL) and lysed in 750 μ L polysome buffer (140 mM KCl, 20 mM Tris-HCl [pH 8.0], 5 mM MgCl₂, 0.5% NP40, 0.5 mg/mL heparin, 1 mM DTT, 100 U/mL RNasin [Promega], 100 μ g/mL CHX). After pelleting, the cytoplasmic lysates were layered onto 11-mL 10%–50% continuous sucrose gradients. The gradients were centrifuged at 35,000 rpm for 2 h at 4°C without brake using a SW40 rotor in a Beckman ultracentrifuge. Afterward, the gradients were collected in 1-mL fractions using a Biologic LP system (Biorad). Absorbance was measured at 254 nm. RNA was precipitated by 1/10 volume sodium acetate (3 M) and 1 volume isopropanol. RNA was further purified using the RNeasy MiniKit (Qiagen) according to the manufacturer's manual. For quality control, equal volumes of RNA of each fraction were analyzed by denaturing agarose gel electrophoresis. RNA was transcribed using the Maxima First Strand cDNA synthesis kit from Fermentas, and subsequently, individual mRNAs were analyzed using real-time PCR with Absolute qPCR SYBR Green Fluorescent mix (Thermo Fisher Scientific). Results are shown as the percentage of mRNA in the single fractions relative to the total amount of mRNA extracted from all fractions. Specific primers were individually designed (gapdh-fwd: TGCACCACTGCTTAGC, gapdh-rev: GGCATGGAC TGTGGTCATGAG; egr2-fwd: ACGTCGGTGACCATCTTTCCCA AT, egr2-rev: TGCCCATGTAAAGTGAAGGTCTGGT; egr3-fwd: GC TTTGTTTCAGTTCGGATCGCCTT, egr3-rev: AAACAATGAGGTG TTTGGG TCGGG; firefly-fwd: ATTTATCGGAGTTGCAGTTGCC CC, firefly-rev: GCTGCGAAATG CCCATACTGTTGA; renilla-fwd: CAGTGGTGGGCCAGATGTAAACAA, renilla-rev: TAAGAAGAGG CCGCGTTACCATGT).

Microarray analysis

For analysis of the polysomal RNA samples, fractions 6–10 from the gradients were pooled. Additionally, total RNA was collected. RNA concentrations were determined using the NanoDrop spectrophotometer (NanoDrop Technologies). RNA quality was assessed using a 2100 Bioanalyzer (Agilent Technologies). RNA was labeled, quality controlled, and hybridized to Illumina HumanHT-12_V3 BeadChip arrays (Illumina, Inc.) at the DKFZ Genomics and Proteomics Core Facility. Data extraction was done for all beads individually, and outliers were removed when MAD (median absolute deviation) > 2.5. All remaining data points were used for the calculation of the mean average signal for a given probe, and standard deviation for each probe was calculated. The statistical analysis was done with the statistical computing environment R version 2.12 (R Development Core Team 2005). Additional software packages were taken from the Bioconductor project (Gentleman et al. 2004).

Western analysis

For Western analysis, cells were lysed and snap-frozen in lysis buffer (50 mM Tris-HCl, 0.27 M sucrose, 1 mM Na-ortho-vanadate, 1 mM EDTA, 1 mM EGTA, 10 mM Na- β -glycerolphosphate, 50 mM NaF, 5 mM Na-pyrophosphate, 1% Triton-X-100, and protease inhibitor mix [Roche]). Fifty μ g of protein were separated on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Proteins were detected using specific antibodies and appropriate secondary antibodies and visualized on an Odyssey infrared imaging system (Li-COR Biosciences GmbH).

RNA affinity chromatography

Egr2-5'UTR (based on NM_000399.3) and partial human reverse gapdh were transcribed in vitro with the MEGAShortscript Transcription kit (Ambion) according to the manufacturer's protocol. The transcript was biotinylated at the 5' end with the 5' EndTag Nucleic Acid Labeling System (Enzo Life Science) according to the manufacturer's instructions. Twenty μ g of biotinylated RNA was conjugated to streptavidin agarose beads in incubation buffer (10 mM Tris-HCl [pH 7.4], 150 mM KCl, 0.5 mM DTT, 0.05% NP40, 100 U/mL RNasin) at 4°C for 2 h with continuous rotation. Five hundred μ g of cytoplasmic proteins of MCF7 cells that were lysed in incubation buffer containing 0.5% NP40 were added to the beads and incubated for 1 h at 4°C followed by 15 min at room temperature. Beads were washed five times with incubation buffer, resuspended in 30 μ L 4 $\times$ Laemmli buffer and boiled for 10 min. Eluted proteins were separated by 10% SDS-PAGE and visualized using silver staining. For MS analysis, SDS gels were stained with Coomassie blue. Corresponding gel lanes were cut in several slices, and proteins were tryptically in-gel digested. Resulting peptides were analyzed by nano-ESI-LC-MS/MS using an LTQ-Orbitrap XL mass spectrometer and an Agilent 1200 nano-HPLC system at the front end. Obtained mass spectra were searched against the species-specific Uniprot protein database (*Homo sapiens*) using the Mascot 2.2 search engine. Peptide matches were filtered by Mascot score cut-off with significance threshold at $P < 0.05$.

Plasmid construction

Primers were designed to amplify the human egr2-5'UTR using RNA extracted from human MCF7 cells (fwd: GGCCGAATTC

GAGCAATTGATAAAGCTCG, rev: TTAACCATGGTT GCTCCT CGCACAAAC). A single product of 326 nt was obtained and inserted into the hairpin-containing bicistronic vector phpRF (kind gift of Prof. Anne Willis) (Coldwell et al. 2000) with SpeI and NcoI, resulting in phpR-egr2-F. Human egr2-5'UTR was also cloned into the promoter-less pGL3-basic vector using HindIII and NcoI, resulting in pGL3-egr2. To rule out cryptic splicing events mediated by the 5' UTR of egr2 in the bicistronic vectors, primers were designed to bind to the 5' end of the *renilla* ORF and the 3' end of the *firefly* ORF (fwd: ATGACTTCGAAAGTTTATGATCCAGAACAAA GGAAACGG, rev: TTACACGGCGATCTTTCCGCCCT). For in vitro transcription, egr2-5'UTR was cloned into the pcDNA3.1(+) vector downstream from the T7-promoter using HindIII and XhoI as described before and linearized with XhoI. pDrive-hr-gapdh was a kind gift of Andreas von Knethen and linearized with NotI.

Reporter assays

MCF7 cells were transiently transfected with the 0.2 μ g DNA using Rotifect reagent (Roth) according to the manufacturer's protocol. After 16 h, the medium was changed, and cells were stimulated. Following stimulation, cells were lysed, and *firefly* and *renilla* luciferase activities were determined using a Dual Luciferase kit assay (Promega) on a Mithras LB 940 luminometer (Berthold). As a transfection control, the SV40- β -Gal vector (Promega) was cotransfected, and β -galactosidase activity was determined using the β -Galactosidase Enzyme Assay System (Promega).

For RNA transfection DNA constructs were linearized with BamHI and in vitro transcribed using the mMACHINE T7 Kit (Ambion) according to the manufacturer's protocol and purified with the MEGAclean Kit (Ambion). Two-tenths μ g of RNA were transfected as described for DNA. Luciferase activities were measured 24 h post-transfection.

Statistical analysis

Each experiment was performed at least three times. Data are presented as mean values $\pm$ SEM. Statistical analysis was performed using Student's *t*-test.

DATA DEPOSITION

The complete gene expression data set has been deposited in the Gene Expression Omnibus under accession no. GSE35022.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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