Pharmacological-based translational induction of transgene expression in mammalian cells

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In the quest for the development of pharmacological switches that control gene expression, no system has been reported that regulates at the translational level. To permit small-molecule control of transgene translation, we have constructed a farnesyl transferase inhibitor-responsive translation initiation factor. This artificial protein is a three-component chimaera consisting of the ribosome recruitment core of the eIF4G1 eukaryotic translation initiation factor, the RNA-binding domain of the R17 bacteriophage coat protein and the plasma membrane localization CAAX motif of farnesylated H-Ras. This membrane-delocalized translation factor is inactive unless liberated in the cytosol. Farnesyl transferase inhibitor FTI-277 prevents the membrane association of the CAAX motif and thus increases the cytoplasmic levels of the eIF4G fusion protein, which is then capable of inducing translation of the second cistron of a bicistronic messenger RNA containing an R17-binding site in its intercistronic space. Such direct translational control by farnesyl transferase inhibitors provides a system for fast, graded and reversible regulation of transgene expression.

Keywords: translation; protein prenylation; inducible gene expression

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INTRODUCTION

The ability to control gene expression is crucial for the production of biological drugs and therapeutic proteins in cell culture as well as in the field of gene therapy. Pharmacological control of transgene expression will enable the development of safe and effective protein delivery methods.

Systems that regulate transcription by means of cell-permeant-inducing agents have been described using tetracycline, macrocyclics, oestrogen, progesterone, isopropyl- β -D-thiogalactoside and

ecdysone (Mills, 2001; Weber & Fussenegger, 2002). A single system targeting a post-transcriptional step of gene expression has been described, which uses aminoglycoside antibiotics to inhibit translation of cognate reporter genes (Werstuck & Green, 1998). However, a strategy that endows induction of gene expression at the post-transcriptional level has not yet been reported.

Recent discoveries on the control of gene expression at the translational level in mammals have shown that tethering the human translation initiation factor eIF4G1 to an mRNA was sufficient to drive productive translation (De Gregorio *et al*, 1999). An initial step in translation initiation involves the recruitment of the 40S ribosomal subunit to mRNAs through interactions with the 5' cap structure and the 3' poly(A) tail (Gray & Wickens, 1998). mRNA sequences called internal ribosome entry sites (IRESs) can also recruit the small ribosomal subunit to a subset of mRNAs (Vagner *et al*, 2001; Bonnal *et al*, 2003). eIF4G1 represents a critical factor in the establishment of interactions between an mRNA and the 40S ribosomal subunit.

We have designed a heterologous translational induction system through small-molecule regulation of the subcellular localization of eIF4G1 (Fig 1). It has been shown that the carboxy-terminal region of eIF4G1 controls translation of reporter genes in mammalian cell culture (De Gregorio *et al*, 1999). By fusing this region of eIF4G1 to the RNA-binding domain of the bacteriophage R17 coat protein, we can specifically control translation of mRNAs containing the 21-nucleotide (nt)-long R17 RNA-binding site (Bardwell & Wickens, 1990), a sequence that is not present in the human genome. Because translation normally occurs in the cytoplasm, sequestering R17–4G to the plasma membrane should block its ability to activate translation of R17 reporter mRNAs. Utilization of the C-terminal region of H-Ras, a domain that is sufficient for plasma membrane localization (Choy *et al*, 1999), enables us to use farnesyl transferase inhibitors (FTIs) to control the localization of eIF4G. The Ras CAAX domain contains consensus motifs for lipid post-translational modifications, including farnesylation and palmitoylation, which promote plasma membrane association. Protein farnesylation involves covalent attachment of the 15-carbon-long farnesyl isoprenoid to the cysteine residue of the C-terminus CVLS sequence. Then, the farnesylated protein undergoes proteolytic cleavage of the VLS

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Fig 1 | Schematic representation of the regulatory principle underlying the pharmacological induction of translation by farnesyl transferase inhibitors.

peptide, carboxymethylation of the farnesylated cysteine residue and attachment of a palmitic acid to an upstream cysteine residue (Zhang & Casey, 1996). As Ras farnesylation is required for oncogenic transformation by Ras, FTIs are being actively studied in research aimed at preventing Ras membrane association and oncogenesis (Sebti & Hamilton, 2000; Singh & Lingham, 2002). In this study, we have successfully used the C-terminal region of H-Ras and the FTI FTI-277 to control the subcellular localization and in turn the translational function of the R17–4G fusion protein.

RESULTS

Membrane-delocalized eIF4G1 is inactive

To test whether expression of R17–4G could induce translation of mRNAs containing the R17-binding site, we constructed plasmids that express haemagglutinin (HA)-tagged fusion proteins between the R17 RNA-binding domain and the eIF4G ribosome recruitment core (amino acids 456–1404) or the NS1 influenza protein, which serves as a negative control (Fig 2A). These plasmids were designed to be used in conjunction with reporter plasmids that encode bicistronic mRNAs coding for the *Renilla* luciferase (LucR) and firefly luciferase (LucF) proteins (Creancier et al, 2000). Whereas translation of the first cistron, LucR, is cap-dependent and is proportional to the amount of RNA produced, translation of LucF is cap-independent and is expected to be very weak in the absence of sequences in the intercistronic space that are able to recruit the 40S ribosomal subunit (De Gregorio et al, 1999). The efficiency of translation of the second cistron, quantified by the ratio of LucF/LucR luciferase activities, was weak for all plasmids producing bicistronic mRNAs regardless of the presence of the R17-binding site in their intercistronic space (Fig 2B). However, the efficiency of translation of the second cistron was increased four- to fivefold in cells transfected with both a reporter plasmid containing an R17-binding site and a plasmid expressing the R17–4G fusion protein (Fig 2B). This effect was specific for reporters containing the R17-binding site, as an increase in second cistron translation was never observed in cells producing R17–4G but transfected with reporters that lack this 21-nt-long sequence. Cells producing R17–NS1, when co-transfected with any of our reporter constructs, showed no LucF translation (Fig 2B). Thus, the R17–4G chimeric protein is sufficient to direct the translation of the downstream cistron of a bicistronic mRNA bearing the R17 protein RNA-binding site in the intercistronic space. Translational induction was observed with the R17-binding site positioned at

either 42 or 186 nt upstream of the LucF initiation codon (Fig 2B), and the presence of multiple R17-binding sites did not enhance this induction (data not shown). As expected, fusion of R17–4G to the 26-residue C-terminal tail of Ras impaired its ability to activate translation of R17-binding-site-containing reporters (Fig 2B). This effect was farnesylation-dependent, as an R17–4G–SVLS fusion protein, in which replacement of the cysteine with a serine impairs farnesylation, activated translation at the same level as the R17–4G protein lacking the CVLS motif (Fig 2B). The presence of the CVLS motif did not affect the expression level of R17–4G, as analysed by western blot (Fig 2C), but rather altered its localization to the plasma membrane (Fig 2D), which was confirmed by direct fluorescence microscopy of cells expressing a yellow fluorescent protein (YFP)–CVLS fusion protein (Fig 2E). These results demonstrate that the R17–4G–CVLS fusion protein was farnesylated, localized at the plasma membrane and consequently not active as an enhancer of translation. Therefore, FTIs can be used as pharmacological inducers of the eIF4G fusion protein and to control translation of specific reporter transgenes.

FTI-277 promotes translation

Human skeletal hepatocarcinoma (SK-Hep1) cells transiently co-transfected with inducer and reporter plasmid were treated with 1 μ M FTI-277 at 24 h after transfection. Whereas this concentration of drug had no effect on the luciferase ratio for cells transfected with the reporter plasmid alone (Fig 3), co-transfection with R17–4G–CVLS specifically enhanced translation of reporter constructs containing the R17-binding site. Addition of FTI-277 did not alter the translation induction property of R17–4G lacking the CAAX domain (Fig 3). No differences in LucR or LucF protein stability were observed after addition of 1 μ M FTI-277 during 8 h (supplementary Fig 1 online), showing that the increase in the LucF/LucR ratio on addition of FTI-277 is indeed due to increased LucF translation.

The translational activation was approximately twofold and was specific for reporter constructs containing an R17-binding site (data not shown). To test the specificity of the action of FTI-277, we generated an R17–4G–CVLS to CVLL mutant that was exclusively geranylgeranylated (Cox et al, 1992), or an SVLS mutant that was unable to undergo prenylation. Whereas the R17–4G and R17–4G–SVLS proteins were predominantly cytoplasmic, the R17–4G–CVLS and R17–4G–CVLL proteins were present in the plasma membrane (Fig 4). Treatment with 1 μ M FTI-277

Fig 2 | Membrane-delocalized eIF4G is not able to promote translation. (A) The chimeric translation factors used in this study contain the HA (haemagglutinin epitope tag) at their amino (N) terminus, the R17 bacteriophage coat protein and the indicated fragments of the human eIF4G, H-Ras and influenza virus NS1 proteins. (B) Translational control by the R17-4G chimeric protein in transiently transfected cells. The cytomegalovirus (cmv) promoter drives expression of bicistronic mRNAs containing a luciferase *Renilla* (LucR) open reading frame (ORF) and a luciferase firefly (LucF) ORF. pCRL-80 and pCRL-188 contain an 80- and a 188-nt-long intercistronic space. pCRL-42R17 and pCRL-186R17 contain an R17 bacteriophage RNA-binding site at 42 and 186 nt upstream of the LucF AUG codon. SK-Hep1 cells were transfected with the pCRL reporter plasmids either alone (mock) or with vectors expressing the R17-4G, R17-4G-CVLS, R17-4G-SVLS or R17-NS1 fusion proteins. Luciferase activities were measured (supplementary Table I online). The translational activity of the second cistron was determined by calculating the LucF/LucR ratio. All the experiments were performed in duplicate on at least five different occasions. (C) Western blotting with a monoclonal antibody against the HA epitope present at the N-terminus of all the R17 fusion proteins. Numbers on the left show molecular masses in kilodaltons. (D) Indirect FITC-labelling immunocytochemistry experiments with the anti-HA antibody on SK-Hep1 cells transiently transfected with the R17-4G and R17-4G-CVLS plasmids. (E) Direct fluorescence experiments on SK-Hep1 cells transiently transfected with the yellow fluorescent protein (YFP)- or YFP-CVLS-expressing plasmids. Scale bar, 50 μ m.

prevented the membrane localization of R17-4G-CVLS but not R17-4G-CVLL. Conversely, 10 μ M of the geranylgeranyl transferase inhibitor GGTI-298 (Miquel *et al*, 1997) prevented the membrane localization of R17-4G-CVLL but not R17-4G-CVLS (Fig 4). These results show that prenyl transferase inhibitors can specifically alter the plasma membrane localization of our eIF4G fusion constructs.

Translational regulation by FTI-277 in stable cell lines

Although the R17-4G-CVLS fusion protein was visualized at the plasma membrane, a significant amount also existed in the cytoplasm. This could be due to the high expression level of the fusion proteins that saturate membrane localization pathways. As a consequence, an unfarnesylated fraction of the fusion protein

remains in the cytoplasm where it can activate translation. This explains the observation that cells co-transfected with pCRL-42R17 and pR17-4G-CVLS have detectable levels of translation of the LucF cistron in the absence of FTI-277 (Fig 3).

To examine the efficiency of our system in the context of lower levels of 4G fusion proteins, stable HeLa cell lines were established. We selected a clone that expressed a low level of R17-4G-CVLS and showed weak translation of the LucF cistron in reporter assays (Fig 5A). Results obtained with this clone are shown in Fig 5. On addition of 1 μ M FTI-277, there is a 6- to 12-fold increase in LucF translation, detectable as early as 2 h and maximally after 8 h of treatment. Again, this effect was specific for reporter constructs containing the R17-binding site and not observed in cells treated with GGTI-298 (Fig 5A). Interestingly,

this effect was reversible. After an 8 h pretreatment with 1 μ M FTI-277, the drug was withdrawn, and between 24 and 48 h later the LucF/LucR ratio returned to uninduced values (Fig 5B). As the rate of accumulation of LucF after FTI-277 treatment is similar to the rate of decay after FTI-277 withdrawal, translation probably stopped immediately when FTI-277 was removed. Thus, FTI-277 is able to strongly and reversibly activate translation of a reporter

Fig 3 | FTI-277-dependent translational induction in transiently transfected cells. SK-Hep1 cells transfected with the pCRL-42R17 alone (mock) or with plasmids expressing either the R17-4G or R17-4G-CVLS fusion proteins were incubated for various durations with FTI-277 (1 μ M). LucF/LucR ratios are shown and were calculated from more than three experiments performed in duplicate. The luciferase activities can be found in supplementary Table II online.

gene containing an R17-binding site in a HeLa cell line that stably expresses low levels of an R17-4G-CVLS fusion protein.

Translation is dose-dependent

To examine the dose responsiveness of FTI-277 and to evaluate further the specificity of the translation induction in our system, we generated a HeLa cell line stably transfected with the pCRL-186R17 reporter plasmid. Subsequent transient transfection with pR17-4G-CVLS showed that an 8 h FTI-277 treatment led to a 10- to 20-fold increase in LucF translation with a reporter containing the R17-binding site (Fig 6). A maximal effect was achieved with a concentration of 1 μ M. Addition of GGTI-298 resulted in a five- to sevenfold increase in translation with the pCRL-186R17 cell line transiently transfected with pR17-4G-CVLL (Fig 6).

Translationally induced cell-cycle arrest

To demonstrate the importance of our system beyond a reporter system, we established inducible cell cycle arrest of Chinese hamster ovary (CHO) cells by regulated expression of the cyclin-dependent kinase inhibitor p27^{Kip1}. Overexpression of p27 results in a G1 arrest in CHO cells and this in turn leads to enhanced protein productivity (Fussenegger *et al*, 1998). To generate an FTI-277-inducible p27 system, we constructed a plasmid that encodes a bicistronic mRNA coding for the truncated mouse MHC class I molecule H-2Kk as a selection marker and for the p27 protein (Fig 7A). CHO cells transiently co-transfected with this plasmid and pR17-4G-CVLS were treated or not with FTI-277 at 24 h after transfection. An 8 h treatment with 1 μ M FTI-277 led to an increase in p27 expression in cells that were selected before analysis for H-2Kk expression on magnetic beads conjugated to a monoclonal antibody against H-2Kk (Fig 7B). By using flow cytometric cell cycle analysis, we detected a significant reduction (from 65% to 42%) of the amount of cells in S phase in FTI-277-treated cells (Fig 7C), thereby showing an FTI-277-dependent growth arrest.

Fig 4 | Prenyl transferase inhibitors relocalize R17-4G-CAAX proteins to the cytoplasm. Immunocytochemistry experiments with the anti-HA antibody in HeLa cells transiently transfected with the R17-4G, R17-4G-CVLS or R17-4G-CVLL expression plasmids and not treated (DMSO) or treated with FTI-277 (1 μ M) or GGTI-298 (10 μ M). Scale bar, 50 μ m.

Fig 5 | Kinetics and reversibility of the translation induction by FTI-277 in stable cell lines. A HeLa cell clone stably expressing the R17-4G-CVLS protein was transiently transfected with pCRL-186R17 (open columns) or pCRL-188 (filled columns). (A) Cells were treated with DMSO, FTI-277 (1 μM) or GGTI-298 (10 μM) for various durations. (B) FTI-277 was removed after 8 h of treatment. Measurements of the luciferase activities (supplementary Table III online) were performed at the indicated times after removal. LucF/LucR ratios were calculated from more than two experiments performed in duplicate.

DISCUSSION

We have demonstrated induction of transgene translation using FTIs. Proof of principle has been obtained in cell culture, but its implementation in mice or in humans is a long way off.

Nevertheless, this class of compounds, under current evaluation as anticancer agents in phase I, II and III clinical trials (Singh & Lingham, 2002), are promising antitumoural agents with minimal toxicity (Johnston, 2001). The bioavailability, low interference with host metabolism and pharmacokinetics of this class of compounds make them a suitable choice for gene therapy applications. We have shown that FTI-277 can induce translation at the nontoxic concentration of 0.1 μM, which is much lower than the amount administered in antiproliferative studies (ranging from 5 to 15 μM depending on the tumour cell line). This low concentration can liberate enough eIF4G fusion proteins to induce efficient translation without perturbing the localization of endogenous farnesylated proteins involved in cell proliferation and transformation. This validates the use of this class of compounds for systems aimed at *in vivo* pharmacological control of gene expression.

The other unique feature of this system that increases its applicability to *in vivo* use is that it is contained in a single vector. The inducer can be encoded by the first cistron and the protein of interest by the second cistron. Our system can also permit inducible coexpression of multiple proteins by using a single plasmid vector. Multicistronic vectors are used at present to produce different subunits of a protein complex (such as IL-12), several co-stimulatory molecules (such as CD70 or CD80) or multiple suicide genes (Wen *et al*, 2001). The ability to control

the expression of a 'cocktail' of proteins is advantageous, as exemplified by the expression of co-stimulatory molecules that enhance the antitumour immune response (Douin-Echinard *et al*, 2000).

By using our system in combination with transcriptional control strategies, gene induction can be controlled at both levels. Thus, leaky transcription can be subsequently controlled at the translational level. In addition, higher levels of induction of gene expression could be achieved when both translation and transcription are simultaneously induced. None of the available inducible systems can claim to reach both the lowest basal level of expression and the highest level of induction, which are aspects required by an ideal inducible system.

METHODS

Plasmid constructs are described in the supplementary information online.

Cell culture, transfection and construction of stable cell lines.

Human skeletal hepatocarcinoma (SK-Hep1) and human cervical epithelial cells (HeLa) were cultured in Dulbecco's modified Eagle's medium (DMEM) or in Ham's F12 for Chinese hamster ovary cells (CHO-K1), supplemented with 10% fetal calf serum and 1 mg/ml G418 (for stable cell clones) in a 10% CO₂ incubator at 37 °C. Transfections were performed with the Fugene 6 reagent (Roche) according to the manufacturer's instructions. GGTI-298 and FTI-277 were purchased from Calbiochem.

Western blotting and enzymatic assays. Cells were harvested 24 h after transfection. Cell lysates were run on a 10% gel and blotted onto nitrocellulose. Immunodetection was performed with

Fig 6 | Translation induction specificity and dose responsiveness by prenyl transferase inhibitors. A HeLa cell clone stably expressing pCRL-186R17 was transiently transfected with the various R17 fusion proteins and not treated (DMSO) or treated with the indicated concentrations of FTI-277 and GGTI-298. LucF/LucR ratios were calculated from more than two experiments performed in duplicate. The luciferase activities can be found in supplementary Table III online.

a monoclonal antibody against the HA epitope (MMS-101R, Eurogentec) or p27 (BD Biosciences) followed by an incubation with a secondary antibody coupled to peroxidase, allowing detection by the chemiluminescence method (ECL kit, Amersham). Luciferase assays were performed with the Dual Luciferase assay kit (Promega) and a Berthold microplate luminometer LB 96V.

Immunocytochemistry. Cellular distribution of R17-4G fusion proteins was studied by both indirect fluorescein isothiocyanate (FITC) labelling and direct fluorescence. Cells were fixed in 3% paraformaldehyde for 7 min. For indirect FITC labelling, cells were permeabilized with 0.025% saponin for 20 min at 37 °C. Non-specific binding sites were blocked by incubation with 0.1 M phosphate buffered saline (PBS), 1% normal goat serum (Tebu), 0.025% saponin and 0.5% bovine serum albumin (BSA) for 40 min at 37 °C. Subsequently, cells were incubated for 2 h with the anti-HA monoclonal primary antibody, followed by a 40 min incubation with an anti-mouse IgG FITC conjugate secondary antibody (Sigma). For direct visualization of YFP by fluorescence, as well as for indirect FITC labelling, cells were examined with a Leitz fluorescence microscope with a $\times 63$ objective.

Cell cycle analysis. Cells were pulsed with 1/100 BrdU (00-0103 ZYMED Laboratories) for the last 20 min, collected and sorted according to the Miltenyi Biotec MACSelect K^K.II Transfected Cell

Fig 7 | FTI-277-based translational induction of p27^{Kip1} leads to reduced cell proliferation. (A) Schematic representation of the bicistronic plasmid encoding the truncated mouse MHC class I molecule H-2Kk and the cyclin-dependent kinase inhibitor p27^{Kip1}. (B) Western blotting with a monoclonal antibody against p27. (C) Percentage of cells in S phase measured by flow cytometric cell cycle analysis.

Selection Kit protocol. After ethanol fixation, immunostaining with 1/300 BrdUrd monoclonal antibody (CHEMICON International) was performed. Fluorescent labelling was performed with an FITC conjugate secondary antibody (1/100). Incubation with propidium iodide (Molecular Probes) was performed and flow cytometric analysis was carried out using an EPICS ALTRA hypersort system coulter (Beckman). Biparametric analysis of total DNA content and BrdU incorporation was performed using the WinMDI 2.8 software. **Supplementary information** is available at *EMBO reports* online (<http://www.emboreports.org>).

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