Agència
de Gestió d'Ajuts
Universitaris
i de Recerca

Memòria justificativa de recerca de les convocatòries BCC, BE, BP, CTP-AIRE, DEBEQ, FI, INEFC, NANOS i PIV

La memòria justificativa consta de les dues parts que venen a continuació:
1.- Dades bàsiques i resums
2.- Memòria del treball (informe científic)

Tots els camps són obligatoris

1.- Dades bàsiques i resums

Nom de la convocatòria

BE

Llegenda per a les convocatòries:

BCC	Convocatòria de beques per a joves membres de comunitats catalanes a l'exterior
BE	Beques per a estades per a la recerca fora de Catalunya
BP	Convocatòria d'ajuts postdoctorals dins del programa Beatriu de Pinós
CTP-AIRE	Ajuts per accions de cooperació en el marc de la comunitat de treball dels Pirineus. Ajuts de mobilitat de personal investigador.
DEBEQ (Modalitat A3)	Beques de Cooperació Internacional i Desenvolupament
FI	Beques predoctorals per a la formació de personal investigador
INEFC	Beques predoctorals i de col·laboració, dins de l'àmbit de l'educació física i l'esport i les ciències aplicades a l'esport
NANOS	Beques de recerca per a la formació en el camp de les nanotecnologies
PIV	Beques de recerca per a professors i investigadors visitants a Catalunya

Títol del projecte: ha de sintetitzar la temàtica científica del vostre document.
Cirp function and characterisation in RNA and microRNAs

Dades de l'investigador o beneficiari

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Paraules clau: cal que esmenteu cinc conceptes que defineixin el contingut de la vostra memòria.
senescence, proliferation, immortalization, cancer, microRNAs, CIRP

Data de presentació de la justificació
12/.3/09

Nom i cognoms i signatura
del/de la investigador/a

Visttiplau del/de la responsable de la
sol·licitud

Resum del projecte: cal adjuntar dos resums del document, l'un en anglès i l'altre en la llengua del document, on s'esmenti la durada de l'acció

Resum en la llengua del projecte (màxim 300 paraules)

With the idea to search for novel genes involved in cell proliferation, we have hypothesized that by infecting primary cells with a cDNA library of immortal cells would render immortalizing genes. Consequently we have discovered CIRP (Cold inducible RNA-binding protein).
Mammalian cells exposed to mild hypothermia show a general inhibition of protein synthesis and a concomitant increase in the expression of a small number of cold-shock mRNAs and proteins. Rbm3, another RNA binding protein belonging to the same family, has been postulated to facilitate protein synthesis at mild cold shock. To investigate if the same occurs for CIRP, CIRP was overexpressed in primary cells and protein sintesis was measured. Interestingly, CIRP increased protein synthesis, however, such increase did not involve an increase in the polysome fraction or affected the ribosome profile.

In addition, the effect caused by CIRP inhibition or knockdown was also analyzed. Different siRNAs against CIRP were tested. Once checked their efficiency by decreasing CIRP at mRNA and protein levels, proliferation was tested by BrdU, cell number (DAPI) and proliferation curves were performed. Interestingly, CIRP provoke a decreased proliferation in primary cells: MEFs, HMEC; and cancer cells: TERA2 and HeLa.
In conclusion, we describe for the first time that CIRP bypasses replicative senescence when over-expressed at physiological temperature (37ºC) by increasing a general protein synthesis. This effect is achieved through ERK1/2 activation in MEFs.
The decrease in growth rate found in mammalian cells treated with mild cold stress is not entirely attributable to arrested metabolism. This decrease may also involve an active process in which CIRP and other stress-responsive proteins play a fundamental role in stimulating proliferation. Although most cell proteins are down-regulated or inhibited with cold stress, CIRP is activated to maintain cells in an active proliferative status and its overexpression at 37°C might be potentially oncogenic.

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2.- Memòria del treball (informe científic sense limitació de paraules). Pot incloure altres fitxers de qualsevol mena, no més grans de 10 MB cadascun d'ells.

Background

The aim of this project was to unravel the characterization of CIRP (Cold induction RNA binding protein) in proliferation. We hypothesize that CIRP might be involved in similar mechanism as Rbm3, another protein belonging to the same family than CIRP and also induced by cold stress. For example, Rbm3 was known to be involved in the following process:

1) Rbm3 binds to 60S ribosomal subunits, alters micro-RNA levels and enhances global protein synthesis.
2) Rbm3 is a potential proto-oncogene as is overexpression stimulates proliferation, a role that is reverted upon its knockdown.

Therefore, we aimed to study CIRP in such biological functions to be performed at Dr. D. Beach Laboratory. Therefore the initially proposed objectives were:

Objectives

1. Study the effect of Cirp overexpression measuring the polysomes fraction by observing the polysome profile and ribosomes from mouse and human cells infected with Cirp versus the empty vector.

2. Design and construction of lentivirus containing siRNA of mouse and human Cirp. Study the effect of siRNA of Cirp in mouse and human models to prove their effect in proliferation. In such models study the reverse effect, if exists over the polysomes profile and ribosomes.

Results

Objective 1.

MEFs and NIH3T3 cells were infected with retrovirus containing CIRP versus vector cDNAs and selected with appropriate antibiotics.
General protein synthesis was measured by ^{35}-S Methionine incorporation in vector versus CIRP infected cells. For that, cells were seeded at a density of 10^5 cells per well of 24 wells. On the next day, ^{35}-S Methionine was added to the cultured media for 24h at 5μCi/ml. Further, cells were washed with PBS and lysed. 50 μl of lysate was applied to GF-C filters to measure ^{35}-S Methionine incorporation by using liquid scintillation spectroscopy. Interestingly, a 2-fold induction of general protein synthesis was observed in CIRP- versus control cells (Figure 1).
Then, the polysome fraction was isolated by spinning the cells at high speed and isolating the supernatant fraction. Such fraction was placed in a sucrose gradient and polysome profile was subsequently measured with a specialized apparatus. No differences were observed between GFP versus CIRP infected cells in the ribosome prolife which included polysomes and a pre-ribosomal fraction corresponding to the microRNAs peak. Therefore the increase in the general protein synthesis induced by CIRP does not involve ribosome biogenesis.

Finally, we analyzed if the microRNAs were affected upon CIRP overexpression. A housekeeping micro-RNA was analyzed. We chose one microRNA that was demonstrated to increase its RNA levels when Rbm2 was pverexpressed designated miR-125b. For this purpose we isolated the micro-RNA fraction in CIRP versus GFP infected cells by using the mir-Vana kit (Ambiom) and then mRNA was amplified using Taqman probes to amplify the microRNA miR-125b. No differences were observed between CIRP versus GFP infected cells in the RNA levels of such microRNA (Figure 2). Thereby we conclude that neither the polysome fraction, neither the microRNAs were generally affected by CIRP overexpression in MEFs or NIH3T3 cells. We did not consider to enlarge our study to additional micro-RNAs as results indicated above were not suggestive of that.

Objective 2.

Three different siRNA against CIRP were purchased from Ambion and assayed in different cellular models, between them: MEFs (Mouse embryonic fibroblast), HMEC (Human mammalian epithelial cells), HMEC-TERT-ER (partially immortalized human mammalian epithelial cells), TERA2 (teratocarcinoma cell line) and HeLa (cancer cell line from the cervix).
Dr Beach´s laboratory has a complete optimization of transient transfection of siRNAs in different cell lines. In addition the role of each siRNA can be perfectly studied and a function assigned in 3-5 days time. Stable infections with the CIRP siRNA were not consider to provide additional information about CIRP proliferative capacity.
Firstly, we checked that different siRNAs against CIRP were able to inhibit CIRP expression at mRNA and protein levels (Figure 3).

Interestingly, we observed that CIRP inhibition reverted the proliferation of CIRP-expressing MEFs indicating that CIRP was the only cause of proliferation induction as its knockdown reverted CIRP phenotype (Figure 4).

Importantly, parallel studies performed at out laboratory (Pathology Department, Hospital Vall d´Hebron) demonstrated that CIRP was overexpressed in a considerable fraction of human tumors; therefore next we assayed CIRP knockdown in several cancer cell lines as TERA2 and HeLa.

Interestingly CIRP inhibition was causing a significant decrease in such cell lines indicating that CIRP behaves as potentially "oncogenic" and therefore similarly to Rbm3 as predicted (Figure 5 and 6).

In order to unravel the mechanism behind CIRP know-down, we tested our initial MEFs model in which we knoew that CIRP indiced P-ERK1/2 expression. Therefore we tested if P-ERK1/2 was inhibited upon CIRP siRNAs in MEFs. Indeed P-ERK1/2 was inhibited in MEFs cells meanwhile decreased proliferation.

In contrast to MEFs cells, neither TERA2 or HeLa modulated P-ERK1/2 in spite of their decreased proliferative capacity.

Conclussions

We identified the CIRP gene by exploiting the entry of MEFs into stress-induced senescence in culture. CIRP over-expression bypasses replicative senescence in primary MEFs. CIRP is a cold-inducible member of the glycine-rich RNA-binding protein family. Although the function

of CIRP has not yet been determined, it has been suggested that this protein may affect gene expression by facilitating translation at mildly cold temperatures, such as 32°C. The increase in total protein synthesis observed in CIRP-MEFs compared to GFP-MEFs prompted a study of translation-related proteins. Our results demonstrated an association between CIRP and an increase in total protein synthesis. These findings agree with a previous study that describes Rbm3, another RNA-binding protein, increasing protein synthesis in a different model of immortalized N2a cells.

In agreement with our previous data, it is important to note that CIRP inhibition not only decreases proliferation in CIRP-MEFs, but also in HeLa cancer cells and the TERA2 cell line.

The P-ERK1/2 status is specifically compromised in MEFs upon CIRP knock-down but not in other cancer cell types tested. Interestingly, although CIRP siRNAs avoid proliferation in such cancer cell lines, CIRP inhibition might affect different target proteins. The fact that the P-ERK1/2 status is not compromised in HeLa or TERA cells when CIRP is inhibited, could be due to the ability of cancer cells to activate simultaneously multiple growth-promoting pathways in order to be fully transformed. Under these circumstances, inhibition of one proliferative-promoting pathway would not compromise their proliferation as other pathways maintain their proliferative status. For example human colorectal cancer cells harbouring a BRAF(V600E) mutation are growth factor independent for the activation of ERK1/2 and survival. Given the fact that CIRP is an RNA binding protein involved in several biological process, its ability to act on different targets might differ in a cell-context-dependent manner.

Our results have demonstrated for the first time a possible CIRP oncogenic function. This is very important because CIRP overexpression at moderate cold-shock induction has a protective effect for the cell (32°C), however its forced overexpression at physiological temperature (37°C) its activation might have drastic consequences in primary cells allowing immortalization.

CIRP and other RNA-binding proteins should be further studied to elucidate their interaction with mRNAs in cell signaling, senescence, and human tumors.

At the moment, the results of this research were completed with previous results from Dr. Lleonart´s team and they are recently published in Molecular and Cellular Biology (Artero-Castro et al., 2009).

Additional work:

Once finished our work in relation to CIRP charaterization, next we performed additional research in relation to the identification of novel microRNAs involved in senescence and immortalization. As the main research line of Dr. Lleonart is the characterization of novel molecular mechanism involved in proliferation, together with the fact that Dr.´s Beach

laboratory has several microRNAs and siRNAs libraries; Dr. Lleonart performed a large-scale microRNA screen.

For such purpose, primary human cells were transiently transfected with a cDNA library of micro-RNAs and those proliferative clones were picked up. Interestingly a total of 28 microRNAs were discovered to stimulate and/or promote proliferation (Figure 7).

Importantly, we were able to demonstrate an implication of the p21 target in this process. Our results establish an important role for the cell cycle inhibitor p21 in growth control of HMEC and extend the repertoire of miRNAs that modulate the activity of this tumor suppressor.

At the moment, results from this additional work have been submitted recently to Cancer Research (Lleonart et al., submitted to Cancer Research; Jan 2009).

Figure 1

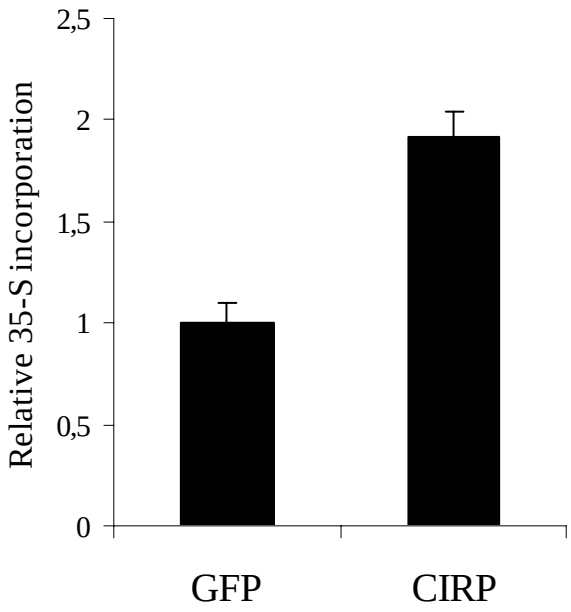

Figure 2

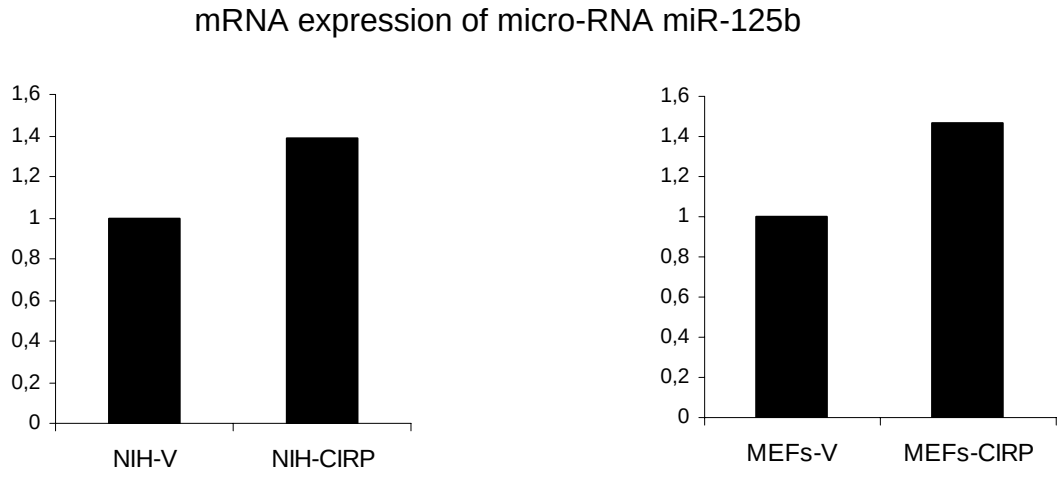

Figure 3

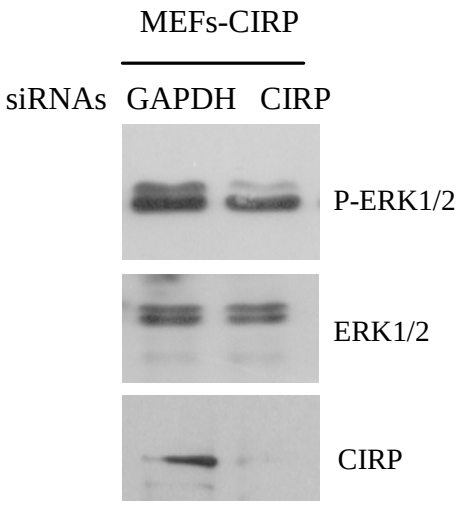

Figure 4

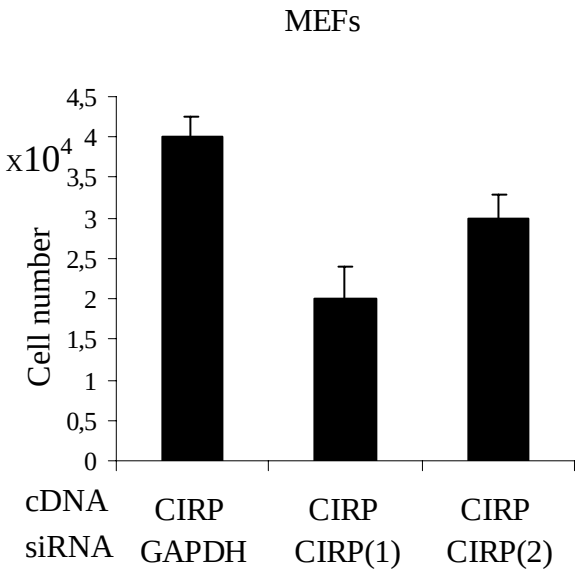

Figure 5

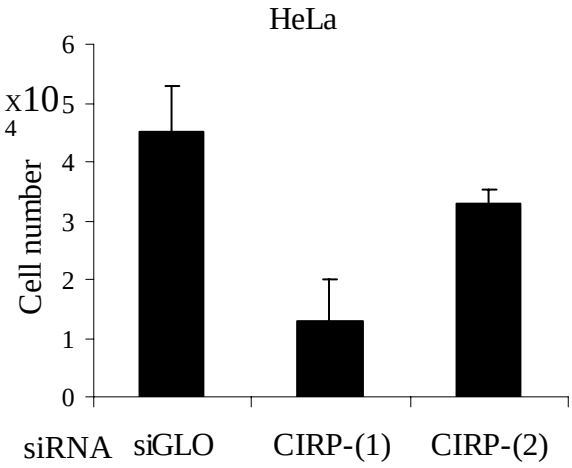

Figure 6

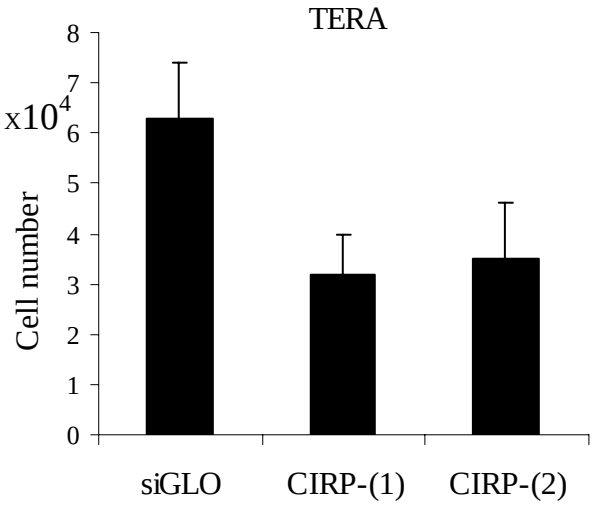

Figure 7