Biochemical and Biophysical Research Communications 443 (2014) 592–597

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Evolutionarily conserved IMPACT impairs various stress responses that require GCN1 for activating the eIF2 kinase GCN2

Tavane D. Cambiaghi [a,1], Catia M. Pereira [a,1], Renuka Shanmugam [b], Michael Bolech [b], Ronald C. Wek [c], Evelyn Sattlegger [b], Beatriz A. Castilho [a,*]

[a] Department of Microbiology, Immunology and Parasitology, Escola Paulista de Medicina, Universidade Federal de São Paulo, Brazil
[b] Institute of Natural and Mathematical Sciences, Massey University, New Zealand
[c] Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, USA

ARTICLE INFO

Article history:
Received 18 November 2013
Available online 11 December 2013

Keywords:
Translational regulation
GCN2
eIF2
GCN1
IMPACT
YIH1

ABSTRACT

In response to a range of environmental stresses, phosphorylation of the alpha subunit of the translation initiation factor 2 (eIF2α) represses general protein synthesis coincident with increased translation of specific mRNAs, such as those encoding the transcription activators GCN4 and ATF4. The eIF2α kinase GCN2 is activated by amino acid starvation by a mechanism involving GCN2 binding to an activator protein GCN1, along with association with uncharged tRNA that accumulates during nutrient deprivation. We previously showed that mammalian IMPACT and its yeast ortholog YIH1 bind to GCN1, thereby preventing GCN1 association with GCN2 and stimulation of this eIF2α kinase during amino acid depletion. GCN2 activity is also enhanced by other stresses, including proteasome inhibition, UV irradiation and lack of glucose. Here, we provide evidence that IMPACT affects directly and specifically the activation of GCN2 under these stress conditions in mammalian cells. We show that activation of mammalian GCN2 requires its interaction with GCN1 and that IMPACT promotes the dissolution of the GCN2–GCN1 complex. To a similar extent as the overexpression of YIH1, overexpression of IMPACT in yeast cells inhibited growth under all stress conditions that require GCN2 and GCN1 for cell survival, including exposure to acetic acid, high levels of NaCl, H_2O_2 or benomyl. This study extends our understanding of the roles played by GCN1 in GCN2 activation induced by a variety of stress arrangements and suggests that IMPACT and YIH1 use similar mechanisms for regulating this eIF2α kinase.

1. Introduction

Translational control of gene expression in eukaryotic cells ensures a rapid response to changing environmental conditions, such as nutrient limitation [1]. An evolutionarily conserved mechanism for sensing the availability of amino acids involves the protein kinase GCN2, which specifically phosphorylates eIF2α, thus lowering protein synthesis. Decreasing protein synthesis conserves cellular nutrients and energy and facilitates reprogramming of gene expression to alleviate nutrient depletion. In mammals, the GCN2 signaling pathway has additionally evolved to control responses in the central nervous system, providing the means to regulate memory and feeding behavior [2–4].

eIF2 is responsible for the delivery of the aminoacylated initiator tRNA to the translational machinery. In this process, eIF2 combines with GTP, which is hydrolyzed to GDP upon recognition of the initiator codon and joining of the large and small ribosomal subunits. To facilitate subsequent rounds of translation initiation, the GDP associated with eIF2 is exchanged for GTP by a guanine nucleotide exchange factor, eIF2B. Phosphorylation of eIF2α on Ser51 blocks this exchange, resulting in the inhibition of general translation initiation [1]. At the same time, translation of specific mRNAs is enhanced by relief of inhibitory upstream open reading frames (uORFs) located in the 5′-portions of targeted transcripts. Among the preferentially translated mRNAs are those encoding GCN4 in yeast and ATF4 in mammals, transcriptional activators that mediate the recovery of cells from the stress insult. Mammals express four eIF2α kinases, GCN2 (here dubbed mGCN2), PERK, HRI and PKR, activated by distinct stress conditions, but some overlap of function may occur [5]. The mechanism of GCN2 activation has been mostly studied in the yeast *Saccharomyces cerevisiae*, where GCN2 (here dubbed yGCN2) is the sole eIF2α kinase [6]. Uncharged tRNAs that accumulate when cells are deprived of amino acids bind to a domain in yGCN2 with homology to histidyl tRNA synthetases (HisRS), altering the conformation of the protein and consequently allowing for yGCN2

* Corresponding author. Address: Departamento de Microbiologia, Imunologia e Parasitologia, Escola Paulista de Medicina, UNIFESP, Rua Botucatu, 862, São Paulo, SP 04023-062, Brazil. Fax: +55 (11) 5572 4711.
E-mail address: bacastilho@unifesp.br (B.A. Castilho).
[1] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.12.021

autophosphorylation and activation. yGCN2 further requires the interaction with an activator protein, yGCN1, with both yGCN2 and yGCN1 being tethered to ribosomes [7–9]. It is proposed that GCN1 is directly involved in transferring uncharged tRNAs that enter the A site of the ribosome to the HisRS-related domain of GCN2 [7,8]. GCN2 is activated by other stresses, that directly or indirectly affect the levels of charged tRNAs.

Two proteins were described that, when overexpressed, inhibit GCN2 activation upon amino acid starvation: YIH1, in yeast, and its mammalian ortholog IMPACT [10,11]. The physiological role of YIH1 for yeast cells is unclear since the deletion of *YIH1* does not increase basal eIF2α phosphorylation. IMPACT is highly abundant in neurons [11–13]. In neuronal cells, endogenous IMPACT lowers basal mGCN2 activation and promotes neuritogenesis, opposing an inhibitory action of mGCN2 [14].

YIH1 and IMPACT share with GCN2 an amino terminal RWD domain that in yGCN2 mediates its interaction with yGCN1 [10,15,16]. YIH1 competes through its RWD domain with yGCN2 for binding to yGCN1, thus lowering yGCN1 association with yGCN2 and reducing activation of this eIF2α kinase [10,17]. IMPACT binds to mGCN1 [11]. However, there is no reported direct experimental evidence that mGCN2 requires binding to mGCN1 for activation.

The present study shows that overexpression of IMPACT inhibits its activation of mGCN2 during several stress conditions in which mGCN1–mGCN2 interaction was found to be required, and we provide evidence that IMPACT disrupts this interaction. We also show in yeast cells that overexpression of IMPACT, to the same extent as YIH1, inhibits growth in all stress conditions that require yGCN2 and yGCN1 function for cell survival, suggesting that IMPACT and YIH1 are functional homologues.

2. Materials and methods

2.1. Plasmids

pIRES-IMPACT (pF281) was constructed by transferring a *Not*I/Klenow/*Eco*RI fragment from plasmid pBE514 [11] into plasmid pIRES-2-EGFP (Clonetech) treated with *Bam*HI/Klenow/*Eco*RI. The sequence of *GCN1* encoding amino acid residues 2204–2651 [11] was introduced into pCDNA3 for expression in mammalian cells (pBE578). Plasmids for the galactose-inducible overexpression of genes in yeast were derived from the plasmid pEMBLyex4 [18], including pES128-9-1 overexpressing GST [8], pES187-B1 overexpressing GST-YIH1 [10] and ES234-6-2 overexpressing GST-IMPACT [11].

2.2. Mammalian cell cultures, transfection and stress conditions

Mouse embryonic fibroblasts (MEF) were cultured as described [11] and transfected at 60% confluency using Lipofectamine (Invitrogen). Six hours later, cells were treated as indicated and extracts prepared as described [14].

2.3. Yeast strains and growth conditions

S. cerevisiae strains used in these studies were strain H1511 (*MATα ura3-52 trp1-63 leu2-3,112 GAL2*$^{+}$ [19] and its isogenic derivatives deleted for *GCN1* (H2556) [8], *GCN2* (H2557) [8] or *YIH1* (ESY11001b) [20]), and strain MSY-WT1 (a *HIS3*$^{+}$ version of BY4741, *MATa leu2Δ0 met15Δ ura3Δ0*) and its isogenic derivatives deleted for *GCN1*, *GCN2,* or *YIH1* (MSY-1-1, EMSY6053-3, MSY-Y2, respectively) [10]. Yeast strains were grown as described [17].

2.4. Immunoblots and immunoprecipitation

Immunoblots were performed as described [14]. Immunoprecipitations were performed as described [11], using 500 μg total protein from cell extracts. Densitometric analyses were performed with Image J.

3. Results and discussion

3.1. Overexpression of IMPACT inhibits mGCN2 autophosphorylation and activation under a variety of stress conditions

Overexpression of IMPACT in MEF cells results in the inhibition of eIF2α phosphorylation elicited by leucine deprivation [11]. To provide more direct evidence that IMPACT inhibits mGCN2 activation, we analyzed the activated status of mGCN2 using antibodies that specifically recognize the phosphorylated Thr898 residue (GCN2-P), which is located in the so-called activation loop of GCN2, a well characterized measure of the activity of this kinase [21–23]. As shown in Fig. 1A, in cells transfected with the vector, the levels of mGCN2-P increase readily after transfer to medium lacking leucine; however, the presence of IMPACT significantly diminished phosphorylation of mGCN2 and of its substrate eIF2α upon amino acid starvation.

We next addressed whether IMPACT inhibits mGCN2 activation by stresses that do not directly involve amino acid depletion, including UV-C irradiation, treatment with the proteasome inhibitor MG132, and glucose deprivation [24–26]. Overexpression of IMPACT resulted in decreased mGCN2 activation and of eIF2α phosphorylation in these additional stress conditions (Fig. 1, panels B, C and D). Although, the extent of inhibition of eIF2α phosphorylation varied between the stresses, IMPACT over-expression led to a decrease in mGCN2-P. We next addressed whether other eIF2α kinases could also be affected by overexpression of IMPACT. Thapsigargin treatment specifically activates PERK by depleting ER calcium stores in the endoplasmic reticulum that elicit a stress in this organelle [27]. Activation of PERK can be monitored by the lower mobility of its autophosphorylated active form during SDS–PAGE [27,28]. In the MEF cells, the presence of IMPACT had no effect on PERK phosphorylation (Fig. 1, panel E). Altogether, these results indicate that IMPACT is a specific inhibitor of mGCN2 under a variety of stress conditions.

3.2. mGCN2–mGCN1 interaction is required for mGCN2 function and is disrupted by IMPACT

In yeast, expression of a portion of yGCN1 (residues 2052–2428), necessary and sufficient to bind to yGCN2, causes a dominant-negative effect, inhibiting yGCN2 [8]. To address whether mGCN1 is an effector of mGCN2, we expressed in MEF cells an analogous fragment of the mGCN1 protein (residues 2204–2651) and determined whether it can also trigger a dominant-negative phenotype [8,11]. The transfected cells expressed two polypeptides that react with the antiserum raised against mGCN1$^{(2204\text{-}2651)}$ [11] (Fig. 2, panel A). The upper band corresponds to the expected size of mGCN1$^{(2204\text{-}2651)}$; the lower band is likely a partial degradation product or a polypeptide initiating at a downstream in frame AUG codon. The cells were then submitted to leucine withdrawal or to treatment with MG132 for the indicated times and cell extracts used for immunoblot analyses (Fig. 2, panel A). Because differences in eIF2α phosphorylation were subtle, we analyzed the downstream effect of mGCN2 activation, i.e., increased expression of ATF4 and its downstream target gene, CHOP, which are induced by and magnify the effect of eIF2α phosphorylation. The presence of mGCN1$^{(2204\text{-}2651)}$ sharply reduced levels of ATF4 and CHOP after

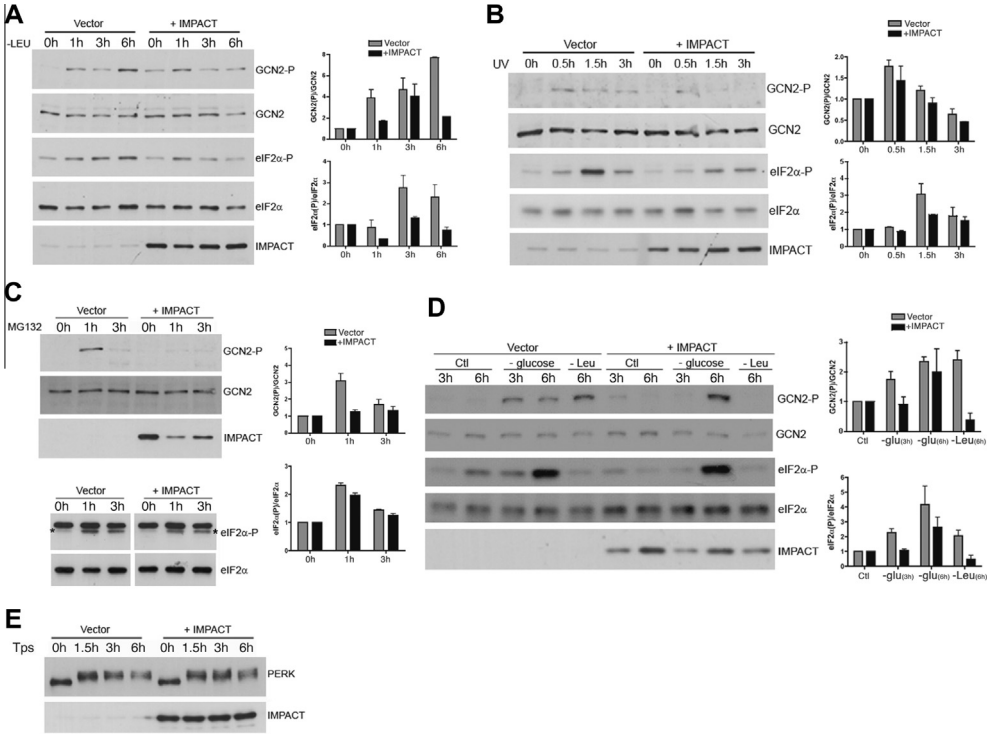

Fig. 1. Specific inhibition of mGCN2 by IMPACT overexpression. (A–D) MEF cells transfected with the vector or with the plasmid expressing IMPACT (+IMPACT) were subjected to (A) leucine starvation, (B) UV irradiation (80 J/m^2), (C) proteasome inhibition (2 μM MG132) or (D) glucose starvation. At the indicated time points, cell extracts were prepared and immunoblots performed using antibodies against IMPACT, and total and phosphorylated (-P) forms of eIF2α and mGCN2. The bands corresponding to eIF2α-P in the bottom C panel are indicated with asterisks. Figures are representative of at least 3 independent experiments. Ratios of phosphorylated/total protein levels for mGCN2 and eIF2α, calculated from at least 3 independent experiments, are indicated in the graphs; the bars indicate +SD. (E) MEF cells transfected with the vector or with the plasmid expressing IMPACT (+IMPACT) were treated with 2 μM thapsigargin for the indicated times and cell extracts analyzed by immunoblots using antibodies against total PERK and IMPACT.

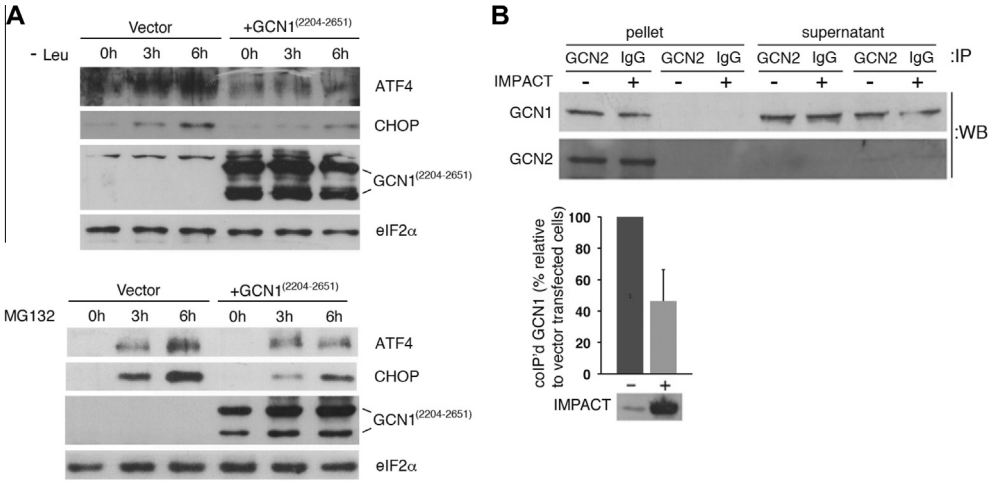

Fig. 2. mGCN1–mGCN2 interaction required for mGCN2 activity is impaired by IMPACT. (A) MEF cells expressing mGCN1$^{(2204\text{-}2651)}$ and control cells transfected with the empty vector where subjected to leucine starvation or to treatment with MG132, and after the indicated times, cell extracts were prepared and immunoblots performed with antibodies against ATF4, CHOP and mGCN1$^{(2204\text{-}2651)}$. Total levels of eIF2α were used for normalization. The figures are representative of at least two independent experiments for each treatment. (B) Extracts (500 μg) of MEF cells transfected with the vector (−) or with the plasmid expressing IMPACT (+) were immunoprecipitated with anti-mGCN2 antibodies or with an irrelevant IgG. The immunoprecipitated material and the supernatant (10%) were subjected to immunoblots with anti-mGCN1 or anti-mGCN2 antibodies. The quantification of mGCN1 associated with mGCN2 from two independent experiments is shown in the graph; the bar indicates +SE. The expression of IMPACT in the cell extracts was determined by immunoblot of the input material using anti-IMPACT antibodies (lower panel).

both treatments, suggesting the attenuation of the translational control directed mGCN2 activation and eIF2α phosphorylation. These results support the idea that activation of mGCN2 by amino acid starvation and proteasome inhibition depends on its interaction with mGCN1.

We then analyzed whether IMPACT competes with mGCN2 for mGCN1 binding by performing co-immunoprecipitation experiments of mGCN2 and mGCN1 in the presence of low or high levels of IMPACT (Fig. 2, panel B). The amount of mGCN1 co-immunoprecipitating with mGCN2 decreased with the overexpression of

IMPACT, as predicted from the yeast model. These observations, taken together, indicate that YIH1 and IMPACT are functionally identical, competing with GCN2 for binding to its activator GCN1.

3.3. Evolutionary conservation of IMPACT function

We previously reported that IMPACT inhibits yGCN2 function under amino acid starvation [11]. YIH1 and IMPACT show high sequence conservation in the C-terminal half [17]. For YIH1, part of the N-terminal half of the molecule mediates its ability to inhibit yGCN2 [15,17]. However, as YIH1 and IMPACT show low sequence conservation in the N-terminal domain, one cannot exclude the possibility that YIH1 and IMPACT diverged during evolution in regards to modulating yGCN2 function. Thus, in order to determine and compare the ability of YIH1 and IMPACT to inhibit yGCN2 under a variety of conditions described to require yGCN2 activation for cell survival, these proteins were overexpressed as GST fusions from a galactose inducible promoter in two yeast strains of different genetic backgrounds, BY4741 and H1511, both lacking endogenous YIH1. We included as control, cells of the same genetic backgrounds devoid of yGCN2. Sensitivity of these yeast strains to a variety of compounds was then investigated. Drug concentrations were adjusted for each background in order to score for phenotypic differences.

3-amino-2,4-triazole (3AT) inhibits histidine biosynthesis, and the resulting activation of yGCN2 is required for cells to grow on 3AT. As reported previously, overexpression of YIH1 or IMPACT impaired cell growth in the presence of 3AT, as found for strains lacking yGCN2 (Fig. 3, A1) [9,11,15]. L-Methionine-S-sulfoximine (MSX), a glutamine synthase inhibitor, activates yGCN2, phosphorylating eIF2α which then promotes cell survival on MSX [10]. YIH1 or IMPACT overexpression caused MSX sensitivity, as expected (Fig. 3, A2). Acetic acid results in intracellular acidification, reducing the activity of aminoacyl-tRNA synthetases leading to yGCN2 activation [29]. The requirement of yGCN2 for cell survival on acetic acid was more evident in the H1511 background. Nevertheless, overexpression of YIH1 or IMPACT led to impaired growth on acetic acid for both strain backgrounds (Fig. 3, A4).

We found in this study that cells require yGCN2 for growth in the presence of the microtubule destabilizing drug, benomyl, a phenotype not described before, and for which no clear link to the levels of aminoacylated tRNAs is currently known. This finding indicates that we still do not know the full suite of GCN2 functions. Importantly, strains overexpressing YIH1 or IMPACT also displayed hypersensitivity to benomyl (Fig. 3, A3).

Overexpression of YIH1 or IMPACT inhibited growth in the presence of high concentrations of NaCl or KCl, similarly to the phenotype of the same strains lacking yGCN2 (Fig. 3, A5,6). It has been described that yGCN2 promotes sodium toxicity [30,31]. However, the lack of yGCN2 in the two genetic backgrounds resulted in increased sensitivity to excess NaCl on SGal medium. Interestingly, when grown on YPD medium, the same strains displayed the reported yGCN2-mediated impairment of growth on high NaCl concentration (Fig. 3, C5). It is noteworthy that

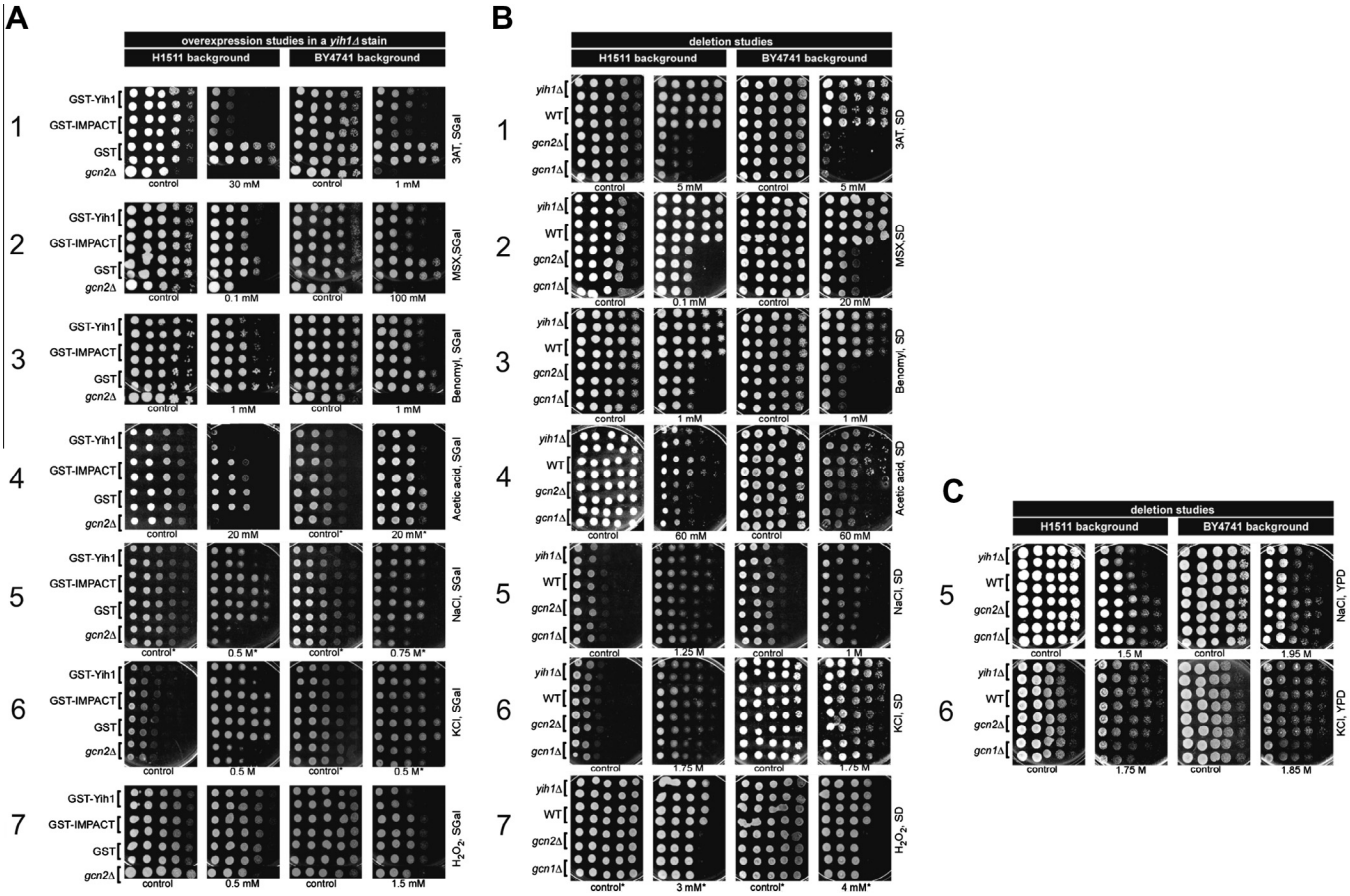

Fig. 3. IMPACT substitutes for YIH1 in inhibiting yGCN1-dependent yGCN2 function under a variety of stress conditions. Cultures of yeast cells, in the H1511 and BY4741 genetic background, grown to saturation, were subjected to 10 fold serial dilutions if not indicated otherwise (*denotes that the overnight culture was diluted 100 fold followed by 2 fold serial dilutions), and 5 μl of each dilution were transferred to indicated medium containing drugs at the indicated concentrations; plates were incubated at 30 °C and growth monitored and documented with a document scanner. (A) Growth on SGal medium of cells overexpressing the indicated proteins in a *yih1Δ* background; (B) growth on SD medium of cells deleted for the genes indicated; (C) growth on YPD medium of cells deleted for the indicated genes.

sensitivity to KCl or to NaCl was affected differently by IMPACT and YIH1 in the two genetic backgrounds. YIH1 overexpression rendered H1511-derived cells sensitive to excess KCl or NaCl, whereas in the BY4742 background, overexpressed IMPACT rendered cells sensitive to excess KCl or NaCl.

yGCN2 has been described to be required for growth in the presence of H_2O_2 [32]. In our assays on SGal medium, however, the effect was subtle. Overexpression of YIH1, but not of IMPACT, increased the sensitivity to H_2O_2 in the BY4741 strain background (Fig. 3, A7). Differences in the strength of YIH1- or IMPACT-mediated yGCN2 inhibition observed between the two backgrounds could be explained by genetic differences in other cellular components affecting the interaction of YIH1/IMPACT with yGCN1. This is supported by previous studies, where we showed that yGCN1 binding to YIH1 is modulated by actin [17]. Strain backgrounds may carry different gene variants that in combination with the amino acid differences between YIH1 and IMPACT may make them more susceptible to either YIH1 or IMPACT function. Alternatively the galactose-induced expression levels of YIH1 versus IMPACT may differ between these strain backgrounds. This is unlikely, however, since the sensitivity to 3AT and MSX conferred by IMPACT or YIH1 overexpression are similar between the two strains (Fig. 3, A1,2).

Taken together the data from both strain backgrounds, in principle, overexpression of YIH1 and IMPACT increased the sensitivity to the same drugs, supporting the notion that these proteins are functional homologues. In addition, overexpression of YIH1 or IMPACT mimics the phenotype of the strains lacking yGCN2, indicating that YIH1 and IMPACT negatively affect yGCN2 function under all stress arrangements tested.

Because the effect of YIH1/IMPACT is predicted to depend on yGCN1 being required for yGCN2 activation, we then studied the effect of the lack of yGCN1 on growth under similar conditions to those used above, in both genetic backgrounds. As expected, both strains were hypersensitive to 3AT, MSX and acetic acid (Fig. 3, B1,2,4). Importantly, yGCN1 was required for resistance to the microtubule destabilizing drug benomyl, indicating that yGCN2 activation by this drug requires yGCN2–yGCN1 interactions (Fig. 3, B3).

yGCN1, as observed for yGCN2, mediates sodium toxicity on YPD medium but sodium resistance on SD medium (Fig. 3, C5). For excess KCl, the absence of yGCN1 in SD medium had a similar, yet subtle phenotype as that seen for the absence of yGCN2 (Fig. 3, B6). But on YPD, y*gcn1Δ* strains displayed increased sensitivity to excess amounts of KCl (Fig. 3, C6). This may suggest that this phenotype is the result of a yGCN1 activity different than its yGCN2-effector function, in agreement with similar predictions published previously [17]. We also show here that yGCN1 is required for H_2O_2 resistance (Fig. 3, B7). Thus, in all cases studied here, both in mammalian cells and in yeast cells, GCN1 is required for GCN2 function, giving support for the idea that the phenotypes of YIH1/IMPACT overexpression result from the disruption of the GCN2–GCN1 interaction.

Although overexpressed YIH1 inhibits yGCN2, the absence of YIH1 does not increase basal levels of eIF2α phosphorylation as detectable on immunoblots of whole cell extracts [10]. Previous attempts at finding a direct phenotype for strains lacking YIH1 had no clear results. Because important strain differences and growth media effects were clear in the assays shown above, we then investigated whether these same strains constructed to lack YIH1 would reveal a phenotype under the conditions used here. These should affect cell growth of *yih1Δ* strains in a manner that is reciprocal to that of *gcn2Δ* or *gcn1Δ* strains.

In the presence of excess NaCl in YPD, strain H1511 lacking YIH1 showed a decreased growth, consistent with the opposite phenotype caused by the absence of yGCN2 and yGCN1 (Fig. 3, C5). This raises the possibility that excess NaCl is a physiological condition that promotes YIH1-mediated yGCN2 inhibition, in order to reduce, to some extent, NaCl toxicity in YPD medium. The absence of YIH1 in the H1511 background caused hypersensitivity to acetic acid (Fig. 3, B4); given that the absence of yGCN2 or yGCN1 also inhibited growth under this condition, this result suggests an additional YIH1 function that is independent of yGCN1/yGCN2. For other growth conditions, the absence of YIH1 did not result in a phenotype. However, other genetic arrangements may uncover other players in the ability of endogenous YIH1 to regulate yGCN2. Indeed, we previously described that a deletion of *YIH1* partially suppressed the inability of cells with a genetic reduction of actin levels to overcome amino acid starvation [10]. It was then suggested that YIH1 resides in a YIH1-actin complex and under specific conditions and/or cellular location YIH1 is released from monomeric actin for inhibition of yGCN2. A localized increase in eIF2α phosphorylation by yGCN2 would not likely be detected by immunoblots of whole cell extracts.

This study showed that in both model systems and in all conditions tested, IMPACT and YIH1 function as inhibitors of GCN2, and that GCN2 requires GCN1 for sensing and overcoming these stress conditions. mGCN1–mGCN2 interaction is then suggested to be integral to mGCN2 function and IMPACT disrupts this interaction, as described for YIH1. The similarities described here between IMPACT and YIH1 provide strong support for the notion that they are functional homologues, allowing further comparative studies. Our novel finding that yGCN2 is required for providing resistance to benomyl indicates that we still do not understand the full range of GCN2 roles.

Acknowledgments

We thank A. Delamano for contributing with experiments in Fig. 2. This work was supported by FAPESP and CNPq grants to B.A.C. and by the Auckland Medical Research Foundation, Maurice & Phyllis Paykel Trust, and Health Research Council Emerging Researcher First Grant to E.S., T.C. and C.M.P. were supported by FAPESP and CAPES post-doctoral fellowships, respectively. R.S. was supported by a Massey University doctoral scholarship, and M.B. by a New Zealand Lottery Health Research PhD scholarship.

References

[1] N. Sonenberg, A.G. Hinnebusch, Regulation of translation initiation in eukaryotes: mechanisms and biological targets, Cell 136 (2009) 731–745.
[2] M. Costa-Mattioli, D. Gobert, H. Harding, et al., Translational control of hippocampal synaptic plasticity and memory by the eIF2alpha kinase GCN2, Nature 436 (2005) 1166–1173.
[3] S. Hao, J.W. Sharp, C.M. Ross-Inta, et al., Uncharged tRNA and sensing of amino acid deficiency in mammalian piriform cortex, Science 307 (2005) 1776–1778.
[4] A.C. Maurin, C. Jousse, J. Averous, et al., The GCN2 kinase biases feeding behavior to maintain amino acid homeostasis in omnivores, Cell Metab. 1 (2005) 273–277.
[5] N. Donnelly, A.M. Gorman, S. Gupta, et al., The eIF2alpha kinases: their structures and functions, Cell. Mol. Life Sci. 70 (2013) 3493–3511.
[6] A.G. Hinnebusch, Translational regulation of GCN4 and the general amino acid control of yeast, Annu. Rev. Microbiol. 59 (2005) 407–450.
[7] M.J. Marton, C.R. Vazquez de Aldana, H. Qiu, et al., Evidence that GCN1 and GCN20, translational regulators of GCN4, function on elongating ribosomes in activation of eIF2alpha kinase GCN2, Mol. Cell Biol. 17 (1997) 4474–4489.
[8] E. Sattlegger, A.G. Hinnebusch, Separate domains in GCN1 for binding protein kinase GCN2 and ribosomes are required for GCN2 activation in amino acid-starved cells, EMBO J. 19 (2000) 6622–6633.
[9] E. Sattlegger, A.G. Hinnebusch, Polyribosome binding by GCN1 is required for full activation of eukaryotic translation initiation factor 2{alpha} kinase GCN2 during amino acid starvation, J. Biol. Chem. 280 (2005) 16514–16521.
[10] E. Sattlegger, M.J. Swanson, E.A. Ashcraft, et al., YIH1 is an actin-binding protein that inhibits protein kinase GCN2 and impairs general amino acid control when overexpressed, J. Biol. Chem. 279 (2004) 29952–29962.
[11] C.M. Pereira, E. Sattlegger, H.Y. Jiang, et al., IMPACT, a protein preferentially expressed in the mouse brain, binds GCN1 and inhibits GCN2 activation, J. Biol. Chem. 280 (2005) 28316–28323.

[12] Y. Hagiwara, M. Hirai, K. Nishiyama, et al., Screening for imprinted genes by allelic message display: identification of a paternally expressed gene *Impact* on mouse chromosome 18, Proc. Natl. Acad. Sci. USA 94 (1997) 9249–9254.
[13] S. Bittencourt, C.M. Pereira, M. Avedissian, et al., Distribution of the protein IMPACT, an inhibitor of GCN2, in the mouse, rat, and marmoset brain, J. Comp. Neurol. 507 (2008) 1811–1830.
[14] M. Roffe, G.N. Hajj, H.F. Azevedo, et al., IMPACT is a developmentally regulated protein in neurons that opposes the eukaryotic initiation factor 2alpha kinase GCN2 in the modulation of neurite outgrowth, J. Biol. Chem. 288 (2013) 10860–10869.
[15] H. Kubota, Y. Sakaki, T. Ito, GI domain-mediated association of the eukaryotic initiation factor 2alpha kinase GCN2 with its activator GCN1 is required for general amino acid control in budding yeast, J. Biol. Chem. 275 (2000) 20243–20246.
[16] H. Kubota, K. Ota, Y. Sakaki, et al., Budding yeast GCN1 binds the GI domain to activate the eIF2alpha kinase GCN2, J. Biol. Chem. 276 (2001) 17591–17596.
[17] E. Sattlegger, J.A. Barbosa, M.C. Moraes, et al., Gcn1 and actin binding to Yih 1: implications of the activation of the eIF2 kinase GCN2, J. Biol. Chem. 286 (2011) 10341–10355.
[18] G. Cesarini, J.A.H. Murray, Plasmid vectors carrying the replication origin of filamentous single-stranded phages, in: J.K. Setlow (Ed.), Genetic Engineering: Principles and Methods, Springer, US, New York, 1987, pp. 135–154.
[19] M. Foiani, A.M. Cigan, C.J. Paddon, et al., GCD2, a translational repressor of the GCN4 gene, has a general function in the initiation of protein synthesis in *Saccharomyces cerevisiae*, Mol. Cell. Biol. 11 (1991) 3203–3216.
[20] T. Waller, S.J. Lee, E. Sattlegger, Evidence that Yih1 resides in a complex with ribosomes, FEBS J. 279 (2012) 1761–1776.
[21] P.R. Romano, M.T. Garcia-Barrio, X. Zhang, et al., Autophosphorylation in the activation loop is required for full kinase activity in vivo of human and yeast eukaryotic initiation factor 2alpha kinases PKR and GCN2, Mol. Cell. Biol. 18 (1998) 2282–2297.
[22] H. Qiu, C. Hu, J. Dong, et al., Mutations that bypass tRNA binding activate the intrinsically defective kinase domain in GCN2, Genes Dev. 16 (2002) 1271–1280.
[23] H.P. Harding, I. Novoa, Y. Zhang, et al., Regulated translation initiation controls stress-induced gene expression in mammalian cells, Mol. Cell 6 (2000) 1099–1108.
[24] H.Y. Jiang, R.C. Wek, Phosphorylation of the alpha-subunit of the eukaryotic initiation factor-2 (eIF2alpha) reduces protein synthesis and enhances apoptosis in response to proteasome inhibition, J. Biol. Chem. 280 (2005) 14189–14202.
[25] H.Y. Jiang, R.C. Wek, GCN2 phosphorylation of eIF2alpha activates NF-kappaB in response to UV irradiation, Biochem. J. 385 (2005) 371–380.
[26] H. Muaddi, M. Majumder, P. Peidis, et al., Phosphorylation of eIF2alpha at serine 51 is an important determinant of cell survival and adaptation to glucose deficiency, Mol. Biol. Cell. 21 (2010) 3220–3231.
[27] H.P. Harding, Y. Zhang, D. Ron, Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase, Nature 397 (1999) 271–274.
[28] K. Ma, K.M. Vattem, R.C. Wek, Dimerization and release of molecular chaperone inhibition facilitate activation of eukaryotic initiation factor-2 kinase in response to endoplasmic reticulum stress, J. Biol. Chem. 277 (2002) 18728–18735.
[29] G. Hueso, R. Aparicio-Sanchis, C. Montesinos, et al., A novel role for protein kinase Gcn2 in yeast tolerance to intracellular acid stress, Biochem. J. 441 (2012) 255–264.
[30] J. Narasimhan, K.A. Staschke, R.C. Wek, Dimerization is required for activation of eIF2 kinase Gcn2 in response to diverse environmental stress conditions, J. Biol. Chem. 279 (2004) 22820–22832.
[31] A. Goossens, T.E. Dever, A. Pascual-Ahuir, et al., The protein kinase Gcn2p mediates sodium toxicity in yeast, J. Biol. Chem. 276 (2001) 30753–30760.
[32] C. Mascarenhas, L.C. Edwards-Ingram, L. Zeef, et al., Gcn4 is required for the response to peroxide stress in the yeast *Saccharomyces cerevisiae*, Mol. Biol. Cell 19 (2008) 2995–3007.