IMPACT, a Protein Preferentially Expressed in the Mouse Brain, Binds GCN1 and Inhibits GCN2 Activation*

Received for publication, July 28, 2004, and in revised form, May 31, 2005
Published, JBC Papers in Press, June 2, 2005, DOI 10.1074/jbc.M408571200

Cátia M. Pereira‡‡, Evelyn Sattlegger†, Hao-Yuan Jiang**, Beatriz M. Longo‡‡‡‡, Carolina B. Jaqueta‡‡, Alan G. Pinhebuchs‡‡, Ronald C. Wek**, Luiz E. A. M. Mello‡‡‡‡, and Beatriz A. Castilho‡‡††

From the Departamentos de Microbiologia, Imunologia and Parasitologia and Fisiologia, Universidade Federal de São Paulo, São Paulo SP 04023-062, Brazil, the Laboratory of Gene Regulation and Development, NICHD, National Institutes of Health, Bethesda, Maryland 20892-2427, and the Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, Indiana 46202

Translational control directed by the eukaryotic translation initiation factor 2 α-subunit (eIF2α) kinase GCN2 is important for coordinating gene expression programs in response to nutritional deprivation. The GCN2 stress response, conserved from yeast to mammals, is critical for resistance to nutritional deficiencies and for the control of feeding behaviors in rodents. The mouse protein IMPACT has sequence similarities to the yeast YIH1 protein, an inhibitor of GCN2. YIH1 competes with GCN2 for binding to a positive regulator, GCN1. Here, we present evidence that IMPACT is the functional counterpart of YIH1. Overexpression of IMPACT in yeast lowered both basal and amino acid starvation-induced levels of phosphorylated eIF2α, as described for YIH1 (31). Overexpression of IMPACT in mouse embryonic fibroblasts inhibited phosphorylation of eIF2α by GCN2 under leucine starvation conditions, abolishing expression of its downstream target genes, ATF4 (CREB-2) and CHOP (GADD153). IMPACT bound to the minimal yeast GCN1 segment required for interaction with yeast GCN2 and YIH1 and to native mouse GCN1. At the protein level, IMPACT was detected mainly in the brain. IMPACT was found to be abundant in the majority of hypothalamic neurons. Scattered neurons expressing this protein at higher levels were detected in other regions such as the hippocampus and piriform cortex. The abundance of IMPACT correlated with yeast GCN2 and YIH1 and to native mouse GCN1. Scattered neurons expressing this protein at higher levels were detected in other regions such as the hippocampus and piriform cortex. The abundance of IMPACT correlated with yeast GCN2 and YIH1 and to native mouse GCN1.

The control of protein synthesis plays an important role in diverse physiological conditions as part of homeostatic mechanisms and long-term memory formation and in pathological conditions such as diabetes, brain ischemia, epilepsy, and other neurodegenerative disorders (1–8). At the cellular level, many signaling networks that affect the rate of protein synthesis involve the phosphorylation of Ser51 in the eukaryotic translation initiation factor 2 α-subunit (eIF2α) by a family of protein kinases that are each activated by different cellular stress conditions.

The heterotrimERIC factor eIF2 is responsible for binding the initiator methionyl-tRNA<sub>Met</sub> in a GTP-dependent mode and delivering it to the 40 S ribosomal subunit. When the initiator AUG codon is encountered, eIF2 is released in a GDP-bound form, with the subsequent formation of the 80 S elongating ribosome. The exchange of GDP for GTP on eIF2 (to allow for further rounds of initiation) is catalyzed by the guanine nucleotide exchange factor eIF2B. Phosphorylation of eIF2α can have a profound inhibitory effect on overall protein synthesis because phosphorylated eIF2α (eIF2α(P)) is a competitive inhibitor of eIF2B, which is limiting in cells (9). Concomitant with this global translation inhibition, eIF2α phosphorylation can lead to preferential translation of mRNAs encoding stress-related proteins. Thus, eIF2α phosphorylation can regulate both general and specific translation.

There are four known eIF2α kinases in mammals (reviewed in Ref. 10): GCN2, activated by amino acid starvation through the binding of uncharged tRNA to its regulatory region; PERK/PKR and eukaryotic translation initiation factor 2 α and eukaryotic translation initiation factor 2 β and eukaryotic translation initiation factor 2 γ (eIF2α and eIF2β and eIF2γ) in eukaryotes. The control of protein synthesis plays an important role in diverse physiological conditions as part of homeostatic mechanisms and long-term memory formation and in pathological conditions such as diabetes, brain ischemia, epilepsy, and other neurodegenerative disorders (1–8). At the cellular level, many signaling networks that affect the rate of protein synthesis involve the phosphorylation of Ser51 in the eukaryotic translation initiation factor 2 α-subunit (eIF2α) by a family of protein kinases that are each activated by different cellular stress conditions. The heterotrimERIC factor eIF2 is responsible for binding the initiator methionyl-tRNA<sub>Met</sub> in a GTP-dependent mode and delivering it to the 40 S ribosomal subunit. When the initiator AUG codon is encountered, eIF2 is released in a GDP-bound form, with the subsequent formation of the 80 S elongating ribosome. The exchange of GDP for GTP on eIF2 (to allow for further rounds of initiation) is catalyzed by the guanine nucleotide exchange factor eIF2B. Phosphorylation of eIF2α can have a profound inhibitory effect on overall protein synthesis because phosphorylated eIF2α (eIF2α(P)) is a competitive inhibitor of eIF2B, which is limiting in cells (9). Concomitant with this global translation inhibition, eIF2α phosphorylation can lead to preferential translation of mRNAs encoding stress-related proteins. Thus, eIF2α phosphorylation can regulate both general and specific translation.

There are four known eIF2α kinases in mammals (reviewed in Ref. 10): GCN2, activated by amino acid starvation through the binding of uncharged tRNA to its regulatory region; PERK/PKR, an endoplasmic reticulum transmembrane protein activated by endoplasmic reticulum stress; PKR, activated by double-stranded RNA produced during viral infection; and HRI (heme-regulated inhibitor kinase), present mainly in reticulocytes and activated by heme deprivation.

GCN2, the sole eIF2α kinase present in the yeast Saccharomyces cerevisiae (11), is found in mice in three isoforms differing only in their N-terminal sequences (12). The most abundant and ubiquitously expressed isoform contains all of the features of the yeast counterpart, including an N-terminal domain, the 5′-terminal domain, the 3′-terminal domain, and the central domain. The 5′-terminal domain contains the consensus sequence for eIF2α phosphorylation in response to amino acid starvation and the 3′-terminal domain contains the consensus sequence for eIF2α phosphorylation in response to double-stranded RNA. The central domain contains the consensus sequence for eIF2α phosphorylation in response to endoplasmic reticulum stress.

The abbreviations used are: eIF2α, eukaryotic translation initiation factor 2 α-subunit; eIF2α(P), phosphorylated eIF2α; PEK/PKR, pancreatic eIF2α kinase/PKR-like endoplasmic reticulum kinase; PKR, protein kinase regulated by RNA; ATF4, activating transcription factor 4; CREB-2, CAMP-responsive element-binding protein-2; CHOP, CCAAT enhancer-binding protein homolog protein; G3P, glutathione S-transferase; 3-AT, 3-aminoatratc; PBS, phosphate-buffered saline; RF, reverse transcription; mGCN1, mouse GCN1; Ni-NTA, nickel-nitrilotriacetic acid; MEF, mouse embryonic fibroblast; yGCN1, yeast GCN1; SCN, suprachiasmatic nuclei.

* This work was supported in part by grants from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (to R. A. and L. E. A. M. M.) and by National Institutes of Health Grant GM49164 (to R. C. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Recipient of a postdoctoral fellowship from FAPESP.

‡ Current address: CPqGM-Fundação Oswaldo Cruz, 40296-710 Salvador, Bahia, Brazil.

‡‡ Recipient of a postdoctoral fellowship from FAPESP. Present address: CPrQGM-Fundação Oswaldo Cruz, 40296-710 Salvador, Bahia, Brazil.

‡‡‡ Supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico.

‡‡‡‡ Supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico. To whom correspondence should be addressed: Dept. Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo, Rua Botucatu, 862, São Paulo SP 04023-062, Brazil. Tel.: 55-11-5576-4537; Fax: 55-11-5572-4711; E-mail: bac@ecb.epm.br.
which is required in vivo for activation of the kinase domain through its interaction with the activator GCN1 (13, 14); a pseudokinase domain followed by the kinase region; a region with similarity to histidyl-tRNA synthetases, implicated in the recognition of uncharged tRNA, which then signals for activation of the kinase domain; and a C-terminal domain, involved in the interaction of GCN2 with the ribosomes (9, 15, 16). GCN1 is required for activation of GCN2, and it is thought that GCN1 acts as a chaperone to transport uncharged tRNAs that enter the A site of ribosomes to the tRNA-binding domain of GCN2 (13, 17). GCN1 works in concert with GCN20 to form a complex with GCN2 on the ribosome, required for activation of the kinase (13, 16, 17).

In yeast, GCN2 is required for growth under amino acid starvation conditions. Its activation by high levels of uncharged tRNAs that accumulate under these conditions leads to eIF2α phosphorylation and thus to the translation of GCN4, a transcriptional activator of hundreds of genes involved in amino acid biosynthesis (18, 19). Mammalian GCN2 has been shown to be required for adaptation to amino acid deprivation in mice and is activated under conditions of low availability of amino acids (20, 21). Although there is no GCN4 ortholog in mammalian cells, the levels of a related transcriptional activator, ATF4, are induced by eIF2α phosphorylation through a mechanism of translation repression similar to that described for yeast GCN4 (22, 23). ATF4 (also known as CREB-2) enhances expression of additional basic leucine zipper transcriptional regulators (including CHOP/GADD153 and ATF6) that together contribute to expression of a large number of genes involved in metabolism, redox chemistry, and apoptosis (21, 24, 25).

Mice lacking GCN2 are viable; however, the GCN2-deficient animals display aberrant behavior in the liver, enhanced skeletal muscle loss, and increased morbidity in response to amino acid starvation (26). Recently, GCN2 has been shown to be directly involved in the feeding behavior of mice. Phosphorylation of eIF2α in the anterior piriform cortex is observed immediately following intake of diets poor in essential amino acids (27). Interestingly, contrary to wild-type animals, which tend to avoid meals lacking one of the essential amino acids, GCN2−/− mice are deficient in this aversive behavior (28, 29).

The GCN2 N-terminal domain has a sequence motif called the GI domain, which is also present in the N-terminal half of the yeast protein YIH1 and its mammalian ortholog, IMPACT (14). IMPACT was originally identified in a screen for imprinted genes in mice. Both YIH1 and IMPACT contain, in the C-terminal half, a conserved sequence (Ancient domain) found also in bacterial proteins (14, 30).

Because of the similarity between the GI domains of YIH1 and GCN2, it has been proposed that YIH1 acts as an inhibitor of GCN2 activation mediated by GCN1 through competition with GCN2 for GCN1 binding. Indeed, recent data in yeast have demonstrated that the binding of GCN2 to GCN1 can be reduced by overexpression of YIH1 and that this leads to reduced eIF2α phosphorylation, indicative of impaired GCN2 activation (31). The in vivo evidence in yeast clearly indicate that YIH1 inhibits GCN2 activation. However, no condition was found in which this inhibitory action would be physiologically relevant to yeast cells because a deletion of YIH1 has no apparent phenotype. Because YIH1 was also found to bind to G-actin, it has been proposed that localized action of YIH1 in yeast cells might regulate the activity of GCN2 in regions where protein synthesis must be maintained at high levels, such as near the growing bud (31).

Given the relevance of GCN2 in mammalian metabolism and behavior and the involvement of eIF2α phosphorylation in several pathological conditions, we decided to study the function of IMPACT. We show here that IMPACT is the mammalian functional counterpart of YIH1. IMPACT binds to GCN1 and acts as an inhibitor of mouse GCN2. We also demonstrate that IMPACT is preferentially expressed in the brain in mice and is especially abundant in the hypothalamus. The levels of IMPACT correlated inversely with the basal levels of eIF2α(Phos) in all tissues examined. Our results strongly suggest that IMPACT acts as an inhibitor of GCN2 in the mammalian brain. These findings have profound physiological implications in the control of phosphorylation of eIF2α and consequently in the expression of ATF4 in different brain areas and specific neuronal cells.

**EXPERIMENTAL PROCEDURES**

**Yeast Methods**—Standard yeast methods were employed (32). Yeast strain H1511 (MATa, uro3-52, trpl-63, leu2-3, leu2-11, GAI1) (33) was grown in synthetic complete medium lacking amino acids to select for plasmids and supplemented with 2% glucose or 10% galactose as carbon source. The plasmid encoding YIH1 fused to glutathione S-transferase (GST) for expression in yeast under the control of the galactose-inducible GAL1 promoter has been described previously (31). The plasmid encoding GST IMPACT fuses to GST under the control of the GAL1 promoter was constructed by introducing BglII and HindIII sites by PCR into the cloned sequence of IMPACT present in plasmid pBE435 (see below) and cloning into the BamHI-HindIII sites of vector pYES28-9-1 as described previously (13). The response of yeast strains to amino acid starvation was studied either by scoring for growth on solid medium lacking histidine and containing 3-aminotriazole (3-AT) at the concentration indicated or by growing cells for 4 h in liquid culture containing 30 mM 3-AT and measuring the levels of eIF2α phosphorylation by immunoblot analysis as described previously (31).

**Animals**—Male Swiss albino mice (20–30 g) were decapitated, and brains and other tissues were removed as quickly as possible, washed with phosphate-buffered saline (PBS), and immediately processed.

**Extract Preparation**—Extracts of whole brain, brain parts, and other tissues were prepared in buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 1 mM phenylmethylsulfonlfuoride, 4 μg/ml aprotinin, 2 μg/ml pepstatin, 100 mM NaF, and 10 mM tetrasodium pyrophosphate. All experiments used independent pools of extracts obtained from four animals.

**RNA Isolation and cDNA Synthesis**—Total RNA from mouse whole brain, cortex, hippocampus, and hypothalamus was obtained by TRIzol extraction as recommended by Invitrogen. For cDNA cloning, reverse transcription (RT)-PCR was performed using the following primer pairs: BC367 (5′-GGCCTTCCTCATGAAGTTTCAGATCG-3′) and BC368 (5′-GGCCGCGTATTAAGATCTTCTCTCTGTCTTCTTC-3′) for IMPACT and BC456 (5′-GGGATCCATGGGCGCCGTTAGGAAGC-3′) and BC457 (5′-GGCTCGAGCGCTGAGCAAGCCGTTCC-3′) for mouse GCN1 (mGCN1; GenBank™ gi:51827542). For detection of transcripts in the brain parts, the following oligonucleotides were used: BC380 (5′-TGCGCTTCTCATAGGATCTGTCATGC-3′) and BC387 (5′-TGCTGCGAGCGCCAGCTGGCTCC-3′) for mGCN1 and BC386 (5′-CAACATACCCACCATGTTCTCCCGAAATAGA-3′) and BC378 (5′-GGGAAGTCCATATCAGCGGAGAG-3′), for βGCN2.

**IMPACT and mGCN1 Cloning and Protein Purification**—A 1-kb DNA fragment comprising the complete open reading frame of IMPACT was obtained by RT-PCR from whole brain RNA using the primers described above. The IMPACT cDNA was inserted as an EcoRI-NotI fragment into the pET22a plasmid (Novagen), generating plasmid pBE435. The N-terminal His-tagged recombinant protein was expressed in E. coli Rosetta (DE3) cells grown in LB medium with 100 μg/ml kanamycin and 100 μg/ml chloramphenicol after induction with 0.1 mM isopropyl β-D-thiogalactopyranoside at 23 °C overnight. Purification of the protein was performed using nickel-nitritoltriacetic acid (Ni-NTA; Qiagen Inc.) essentially as recommended by the manufacturer. Briefly, the cells were harvested, resuspended in lysis buffer (10% sucrose, 0.2 M NaCl, and 50 mM Tris-HCl (pH 7.5), frozen, incubated with lysozyme (1 mg/ml) for 30 min on ice, and briefly sonicated. The cell lysates were centrifuged, and the supernatant was applied to Ni-NTA equilibrated with binding buffer (500 mM NaCl, 20 mM Tris-HCl (pH 8.0), and 5 mM imidazole). The column was washed with washing buffer (500 mM NaCl, 20 mM Tris-HCl (pH 8.0), and 20 mM imidazole). The His-tagged protein was eluted with elution buffer (500 mM NaCl, 20 mM Tris-HCl (pH 7.5) and 10 mM 2-mercaptoethanol. The mGCN1 sequence (GenBank™ gi:51827542) encoding residues 2204–2651 was obtained.
by RT-PCR and cloned as a 1.3-kb BamHI-XhoI fragment into plasmids pET28a and pGEX6p3. Expression was carried out in E. coli BL21 (DE3) cells for the His-tagged protein and in DH5α cells for the GST fusion. Bacterial extracts were prepared as described above. The insoluble recombinant proteins present in the bacterial extract pellet were solubilized by 8 M urea. The His6-mGCN1 protein used for immunization was purified on Ni-NTA in the presence of urea as recommended by the manufacturer, followed by preparative SDS-PAGE and elution from the gel. The GST-mGCN1 protein was purified from the urea-solubilized pellet by preparative SDS-PAGE, followed by elution of the protein from the polyacrylamide gel slice.

Mouse Embryonic Fibroblast (MEF) Cell Transfection and Amino Acid Starvation Conditions—For overexpression of IMPACT in MEF cells, an EcoRI-NotI fragment encoding IMPACT was isolated from plasmid pBEE435 and placed under the control of the cytomegalovirus promoter in plasmid pCI-neo (Promega), which contains the SV40 origin of replication, originating plasmid pBHE514. MEF cells immortalized by the SV40 large T antigen were cultured in Dulbecco’s modified Eagle’s medium (BioWhittaker, Inc.) supplemented with 1 mM nonessential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum and transfected using Lipofectamine (Intron) at 50 °C for 30 min, the same membrane was incubated with 5% nonfat milk in PBS (for anti-IMPACT antibodies) or in Tris-buffered saline, 1% bovine serum albumin, and 0.1% Tween 20 for 1 h at room temperature, followed by overnight incubation with 5% nonfat milk in PBS, followed by incubation with antiserum at a 1:10 dilution in PBS for 3 h. After washing with PBS, bound antibodies were eluted with 0.1 M glycine (pH 2.5) and 1 mM EGTA for 10 min. The pH was immediately neutralized by adding an equal volume of 0.1 N Trizma (Tris base).

Immunoblot Analysis—For immunoblot analysis of IMPACT expression and eIF2α phosphorylation, Laemmli sample buffer was added to the samples, and after boiling for 3 min, the proteins were separated on 12% SDS-polyacrylamide gel and transferred to nitrocellulose membrane (Hybond-C Extra, Amersham Biosciences) at 1 A for 1 h using the manufacturer. The purified proteins were dialyzed against 20 mM Tris-HCl (pH 8.0) and 1 mM EDTA. For the pull-down assays, the purified proteins (20 μg) were immobilized on 20 μl of glutathione-Sepharose beads and incubated with 500 μg of brain extract prepared as described above in a total volume of 200 μl in 30 mM Tris-HCI (pH 7.5), 50 mM KCl, 10% glycerol, 0.1 mM phenylmethylsulfonfyl fluoride, and 5 mM β-mercaptoethanol for 2 h at 4 °C. After washing with PBS, the beads were resuspended in 20 μl of 4% Laemmli sample buffer and boiled for 5 min, and the proteins were separated on 12% SDS-polyacrylamide gel. After transfer to a Hybond-C membrane, the proteins were stained with Ponceau S. Western blotting using anti-IMPACT antibodies was performed as described above.

Co-immunoprecipitation of mGCN1 and IMPACT—Brains (5 mg) were precleared by incubation with 20 μl of protein A-agarose beads and 1 μl of preimmune serum in the buffer described above for the preparation of extracts from mouse tissues. The supernatant was then incubated overnight at 4 °C with 20 μl of protein A-agarose bead-bound anti-mGCN1 antibodies, an irrelevant IgG, or buffer only. The beads were washed three times with the same buffer, and the bound material was resolved by SDS-PAGE, followed by immunoblotting using anti-IMPACT or anti-mGCN1 antibodies.

Immunohistochemistry for IMPACT—Animals were deeply anesthetized with a thionembutal overdose (100 mg/kg) and perfused through the heart with 50 ml of saline, followed by 300 ml of 4% paraformaldehyde at 4 °C. The brains were removed and cryoprotected in 30% sucrose in PBS for 24 h. Coronal sections (40 μm thick) were collected, washed with PBS for 30 min, and incubated in blocking buffer (PBS containing 0.5% Triton X-100 and 0.5% normal goat serum (Vector Laboratories)) for 30 min, followed by incubation with anti-IMPACT monoclonal antibodies (diluted 1:100 in blocking buffer) at room temperature for 24 h. The sections were then washed with PBS for 30 min, incubated with goat anti-rabbit biotinylated IgG (1:200 dilution) for 2 h, washed again with PBS for 30 min, and incubated with avidin-biotin complex (Elite ABC kit, Vector Laboratories) for 90 min. The bound antibodies were detected with a nickel-intensified diaminobenzidine tetrahydrochloride reaction.

**FIG. 1. Overexpression of IMPACT in yeast causes a Gen- phenotype.** A, serial 10-fold dilutions of overnight cultures from strain H1511 expressing GST-IMPACT (two independent transformants are shown), GST-YTH1, or GST under the control of the galactose-inducible promoter from plasmids pES234-6-2, pES187-B1, and pES128-9-1, respectively, were grown on minimal medium containing galactose alone or supplemented with 30 mM 3-AT. B, shown are the results from immunoblot analysis of whole cell extracts (4 μg) prepared from the same strains shown in A grown in galactose using anti-GST serum (upper panel). The S22 ribosomal protein was used for normalization (lower panel).
RESULTS

Overexpression of IMPACT in Yeast Cells Causes a Gcn− Phenotype—To determine whether IMPACT is functionally related to YIH1, we overexpressed this protein in yeast cells. Overexpression of YIH1 imparts a Gcn− phenotype to yeast cells (31), which can be identified by the inability to grow in the presence of 3-AT, an inhibitor of the HIS3 enzyme necessary for the biosynthesis of histidine. To grow in the presence of 3-AT, cells have to synthesize GCN4, a transcriptional activator of amino acid biosynthetic genes and salvaging of nutrients. GCN4 is under translational regulation and is synthesized under conditions that lower the amounts of active ternary complex, such as when eIF2α is phosphorylated. Thus, cells unable to phosphorylate eIF2α will not translate GCN4 and therefore will not overcome the inhibitory action of 3-AT, visible by impaired growth. To assess the effect of IMPACT on the ability of cells to grow in 3-AT, IMPACT was expressed in yeast as a fusion with GST under the control of the galactose-inducible promoter. Cells overexpressing GST-YIH1, also under the control of the galactose-inducible promoter, were used as a control. Cells overexpressing IMPACT displayed a Gcn− phenotype, i.e. impaired growth in the 3-AT-containing medium, as did cells overexpressing YIH1 (Fig. 1A). The levels of both proteins were similar, as shown by immunoblotting performed in extracts prepared from galactose-induced cultures using antibodies directed against GST (Fig. 1B).

The Gcn− Phenotype of IMPACT Overexpression in Yeast Is Due to Inhibition of eIF2α Phosphorylation—The Gcn− phenotype is an indication of a defect in eIF2α phosphorylation and GCN4 translational derepression, such as in cells with a deletion of the eIF2α kinase GCN2. We then investigated the levels of eIF2α phosphorylation in cells overexpressing IMPACT. There is a basal level of eIF2α phosphorylation even under optimal growth conditions, which is then significantly elevated when cells are grown under amino acid starvation conditions or elicited by the addition of 3-AT. As shown in Fig. 2, the basal levels of eIF2α phosphorylation were decreased in cells overexpressing IMPACT (compare lanes 2 and 3 with lane 4), similar to the effect observed for YIH1 overexpression (compare lane 1 with lanes 2 and 3). IMPACT overexpression also hindered activation of GCN2 under starvation conditions (lanes 6 and 7 versus lanes 8–10) to the same extent as did YIH1 overexpression, lowering eIF2α phosphorylation levels to approximately half of the eIF2α(P) levels found upon GST overexpression. These data thus indicate that IMPACT inhibits GCN2 activation in yeast, probably by interacting with and sequestering yGCN1.

Overexpression of IMPACT in MEFs Inhibits GCN2 Activation upon Leucine Starvation—GCN2 is required for induced eIF2α phosphorylation and enhanced expression of ATF4 and its target gene CHOP in response to amino acid limitation in mammalian cells (21, 25). To determine whether IMPACT inhibits mammalian GCN2, MEF cells were transfected with a plasmid expressing IMPACT under the control of the cytomegalovirus promoter. There were high levels of IMPACT in the transfected MEF cells as judged by immunoblot analysis compared with the cells carrying only the vector or the non-transfected cells (Fig. 3B). Activation of GCN2 was determined by phosphorylation of eIF2α upon incubation of the cells in medium lacking leucine. The non-transfected cells showed a significant increase in eIF2α(P) that was the result of activation of GCN2, as illustrated by the observation that eIF2α(P) levels were diminished in GCN2−/− cells that were subjected to leucine starvation (Fig. 3A). Cells carrying the vector alone showed an increase in the basal levels of eIF2α(P) in the presence of leucine compared with the non-transfected cells, suggesting perhaps that the transfection procedure and/or high replication rate of the plasmid activates an eIF2α kinase; however, activation of GCN2 upon leucine starvation was clearly evident in these cells. Although displaying the same amount of basal eIF2α(P) as the vector-containing cells under non-starvation conditions, MEF cells overexpressing IMPACT clearly did not show the same increase in eIF2α(P) levels upon leucine depletion (Fig. 3B). The signal for eIF2α(P) remained constant throughout the 6 h of incubation without leucine. Given that eIF2α(P) signals for the increased expression of ATF4 and CHOP, we also investigated whether overexpression of IMPACT affects the levels of these two proteins. Consistent with an earlier report (25), translational induction of ATF4 expression contributed to early expression of this transcriptional regulator, within 1 h of leucine limitation in the wild-type or vector-transfected MEF cells. The levels of the ATF4 target gene CHOP were also induced in these cells within 3 h of leucine starvation. Notably, overexpression of IMPACT resulted in minimal production of ATF4 and CHOP upon amino acid starvation compared with the cells transfected with the vector (Fig. 3B). These results clearly show that IMPACT inhibits activation of the mammalian GCN2 stress pathway in response to nutrient deprivation.

IMPACT Binds Yeast and Mammalian GCN1—To determine whether IMPACT inhibits GCN2 by binding to GCN1, as does its yeast ortholog, we first addressed whether IMPACT could bind yGCN1. It has been shown that residues 2052–2428 of yGCN1 are sufficient for interaction with both yeast GCN2 and YIH1 (13). The region comprised by residues 2052–2428 of the yeast protein shows 34% identity and 61% similarity to the equivalent region in mGCN1. The mutation R2259A in this region has been shown to abolish the interaction of yGCN1 with both yGCN2 and YIH1 when present in the complete protein in vitro and in a GST-yGCN1-(2052–2428) fusion protein in vitro (13, 31). Arg2259 is identical in all neighboring sequences or highly similar in all sequenced orthologs, as shown in the alignment of Fig. 4A. We then used the purified GST-yGCN1-(2052–2428) fusion protein in pull-down experiments with extracts prepared from mouse brain (see below). As shown in Fig. 4B, brain IMPACT was immobilized on glutathione beads through interaction with GST-yGCN1-(2052–2428). Because only a small fraction of IMPACT present in the brain extract associated with the immobilized GST-yGCN1 protein (−0.2−0.5%), we used also the yGCN1 mutant in which residue 2259 was altered from arginine to alanine (GST-yGCN1-(2052–2428)R2259A) to address the specificity of binding. This mutant protein was unable to bind to IMPACT.
FIG. 3. Inhibition of GCN2 activation by IMPACT in mammalian cells. A, GCN2+/+ and GCN2−/− MEF cells were subjected to leucine starvation conditions for the indicated number of hours (3 and 6 h) or to no stress (0 h), and immunoblot analyses were carried out to measure activation of the GCN2 stress pathway. B, non-transfected GCN2+/+ MEF cells or GCN2−/− MEF cells transfected with the pCI-neo plasmid vector alone or with the plasmid expressing IMPACT were grown in medium lacking leucine for the indicated number of hours (1, 3, and 6 h) or with no stress (0 h). Equal amounts of protein lysates were analyzed by immunoblotting using antibodies against IMPACT, CHOP, ATF4 eIF2α(P), or β-actin.

FIG. 4. IMPACT binds yeast and mammalian GCN1. A, conservation of GCN1 sequences. Shown is the alignment of the region of GCN1 around Arg2259 (numbering relative to the yeast protein) (arrow) from Mus musculus (Mm; GenBankTM gi:51827542), Homo sapiens (Hs; gi:41148981), S. cerevisiae (Sc; gi:477122), Neurospora crassa (Nc; gi:32410555), and Arabidopsis thaliana (At; gi:5042415), with identical residues indicated in reverse boldface and conserved residues boxed. B, GST pull-down. Wild-type yGCN1-(2052–2428) (R2259) and mutant yGCN1-(2052–2428) (R2259A) (A2259) fused to GST and GST alone (20 μg each) purified from E. coli were incubated with 20 μl of glutathione-Sepharose beads and with 500 μg of whole extracts from mouse brains. The proteins associated with the beads (100% of the bound material; pellet lanes) were subjected to SDS-PAGE and detected by immunoblotting with monospecific antibodies raised against IMPACT (upper panel) and by Ponceau staining (lower panel). The input lanes correspond to one-fiftieth of the extract used in the pull-down assay. C, co-immunoprecipitation. Protein A-agarose beads alone (−) or coupled to monospecific antibodies raised against mGCN1 or to an irrelevant IgG were incubated with total brain extracts. Alternatively, it is possible that the native IMPACT protein is present in a large complex that may not be stable enough to be retained by the GST-yGCN1 beads.

The small amount of binding observed for the wild-type GST-yGCN1-(2052–2428) protein could be due to the use of a fragment of a heterologous protein that may bind with low affinity to the endogenous IMPACT protein present in the extracts. Alternatively, it is possible that the native IMPACT protein is present in a large complex that may not be stable enough to be retained by the GST-yGCN1 beads. To show that IMPACT interacts with mGCN1, we raised antibodies against part of the mGCN1 protein (residues 2204–2651, containing the putative GCN2/IMPACT-interacting region) for use in a co-immunoprecipitation assay. The complete sequence of mGCN1 cDNA indicates a protein of 2806 residues, with a predicted mass of 307 kDa. These antibodies recognized a protein of −280 kDa and, to a lesser degree, a slower migrating protein; the latter was also recognized by the preimmune serum. The smaller than predicted mass was also observed for the native yGCN1 protein. As shown in Fig. 4C, IMPACT was immunoprecipitated along with mGCN1 from brain extracts using the purified antibodies. mGCN1 and IMPACT were not detected in the control, in which unrelated purified IgG was used in the same amount as the purified anti-mGCN1 antibodies. Taken together, these results provide strong evidence that IMPACT binds specifically to GCN1.

A very small percentage of IMPACT was found associated with GCN1 in brain extracts as detected by this co-immunoprecipitation assay. This result is not surprising given that a more extensive interaction would probably block the amino acid starvation response mediated by GCN2. Interestingly, in the yeast model, the in vivo interaction between YIH1 and yGCN1 can be detected only when YIH1 is overexpressed (31). Thus, it is possible that the IMPACT-GCN1 complexes occur exclusively in a small population of neuronal cells that express high levels of IMPACT.

IMPACT Is Preferentially Expressed in the Hypothalamus—It has been previously shown through in situ and Northern hybridizations in mice that the IMPACT mRNA is preferentially expressed in the brain (30). Using highly specific antibodies directed against IMPACT raised in this study, we analyzed in detail the abundance of this protein in mouse tissues. Immunoblots of extracts obtained from several organs showed that the IMPACT pro-
protein were loaded (30, 15, 10, and 5 used to normalize the amounts of total protein added to each lane by hippocampus, and hypothalamus (Fig. 5 titrated by Western blotting of extracts prepared from the cortex, brain areas (35). These results were further confirmed and quan-
pared with other organs and where the hypothalamus had 10 times more IMPACT mRNA com-
sion Database (available at expression.gnf.org/cgi-bin/index.cgi), agreement with data obtained from the Mouse GNF Gene Expres-
sional, and posterior hypothalamic nuclei; the preoptic area; and the third ventricular region, including the paraventricular, dorsome-
hippocampal regions. Strong labeling was evident around the intense labeling. Expression in other neuronal cells in these regions
scattered neurons in several brain areas, as shown here for the
in Fig. 6, IMPACT was found to be expressed at high levels in some
performed using mouse brain slices. As a control, we used nonspe-
expression in different brain areas, immunohistochemistry was
carried out RT-PCR analysis for the GCN1 transcript in the
transcriptome microarray data base (35), and we therefore
carried out RT-PCR analysis for the GCN1 transcript in the
different brain areas. The results indicate that both βGCN2 and
GCN1 are expressed in the hypothalamus, cortex, and hippocampus (Fig. 7B). Thus, the different basal levels of
eIF2α(β) in these areas might be related to the levels of IM-
Gn2 and thus to the differential basal activation of GCN2.
To provide another comparative analysis, we also quanti-
ted the levels of eIF2α(β) in the heart, where there is little
IMPACT protein. Basal eIF2α(β) levels were found to be very
high in the heart compared with those in the hypothalamus
(Fig. 7C), therefore providing further support for an inverse
relationship between the abundance of IMPACT and eIF2α

**DISCUSSION**

It has been recently shown that YIH1 regulates activation of the eIF2α kinase GCN2 in yeast through its interaction with GCN1 (31). In this study, we have provided *in vivo* evidence that IMPACT, the mammalian ortholog of YIH1, inhibits both yeast and mouse GCN2 by its ability to bind to GCN1. We suggest that IMPACT is a negative regulator of GCN2 in mammals.

As a prediction of this hypothesis, high levels of IMPACT should lead to low intrinsic activation of GCN2 in mouse tis-
sues and therefore to lower basal levels of eIF2α phosphor-
ylation, as observed in the yeast model. We were able to show that this correlation occurred in all of the mouse tissues tested. In the heart, where almost no IMPACT could be detected relative to the brain, high levels of eIF2α(P) were found. In the hypo-
thalamus, where IMPACT was expressed, basal levels of eIF2α(P) were much lower in the hypothal-
amus, where high amounts of IMPACT were found, than in the hippocampus and cortex.

It was possible that the low eIF2α(P) levels in the hypoth-
amus could be instead due to lower levels of GCN2 or GCN1. In
the mouse transcriptome microarray data base (35), GCN2 was found to be equally expressed in all of the brain areas analyzed here. However, the microarray data did not differentiate among the three isoforms of GCN2 present in mice, all of them known to be expressed in the brain (12). The β-isoform has complete homology to other GCN2 homologs, including the region at the N terminus that may interact with GCN1. The α-isoform lacks the N-terminal 280 amino acid residues and is therefore not a target for GCN1 binding. The γ-isoform starts at an amino acid corresponding to position 86 of βGCN2 and carries six additional residues in the N terminus. This isoform contains part of the GI domain. To investigate the presence of the βGCN2 isoform in the different brain areas, we performed RT-PCR using oligonucleotides specific for this isoform. Furthermore, GCN1 mRNA was not reported to be present in the transcriptome microarray data base (35), and we therefore
carried out RT-PCR analysis for the GCN1 transcript in the
different brain areas. The results indicate that both βGCN2 and
GCN1 are expressed in the hypothalamus, cortex, and hippocampus (Fig. 7B). Thus, the different basal levels of
eIF2α(P) in these areas might be related to the levels of IM-
Gn2 and thus to the differential basal activation of GCN2.
To provide another comparative analysis, we also quanti-
ted the levels of eIF2α(P) in the heart, where there is little
IMPACT protein. Basal eIF2α(P) levels were found to be very
high in the heart compared with those in the hypothalamus
(Fig. 7C), therefore providing further support for an inverse
relationship between the abundance of IMPACT and eIF2α
phosphorylation.

**The Hypothalamus Displays the Lowest Basal Levels of eIF2α Phosphorylation**—Because our results showed that IMPACT overexpression inhibited mouse GCN2, we hypothesized that the high levels of IMPACT in the hypothalamus may inhibit endogenous GCN2, resulting in low basal levels of eIF2α phos-
phorylation in this brain region compared with other areas with low IMPACT expression. We then determined the ratio of eIF2α(P) to total eIF2α by immunoblotting of extracts from the cortex, hippocampus, and hypothalamus. As shown in Fig. 7A, the basal levels of eIF2α(P) were much lower in the hypothal-
amus, where high amounts of IMPACT were found, than in the hippocampus and cortex.
of a normal animal should rely heavily on GCN2 and PEK/PERK, both of which are intrinsically related to metabolic regulation. Low glucose activates PEK/PERK and possibly GCN2, in analogy to yeast (36) and from the phenotypes of knockout animals suggesting an overlap of PEK/PERK and GCN2 in glucose sensing, whereas amino acid deprivation ac-
IMPACT, a GCN2 Inhibitor in Mammals

28323

activates GCN2. Both mechanisms are necessary for maintaining homeostasis and should be constantly monitored. Thus, GCN2 activity can be considered as an important contributor to the levels of phosphorylated eIF2α in mammals under normal physiological conditions. The results shown here strongly suggest that, under amino acid starvation conditions, IMPACT has an important role in controlling the levels of eIF2α(P) through GCN2 inhibition, which may be more relevant in specific populations of neuronal cells.

The hypothalamus is critically involved in the maintenance of homeostasis, such as the control of body temperature and the balance of fluids and energy, and it is constantly adjusting the organism’s metabolism and behavior to its immediate needs. It is interesting to speculate that, due to the constant signaling required from neurons in the hypothalamus, protein synthesis must be maintained at constant high levels even under conditions in which GCN2 would be activated in other cell types, such as amino acid starvation. Thus, the mechanism of inhibition of GCN2 activation represented by overexpression of IMPACT may be important for the function of neurons in this area. Along these lines, it is possible that an inhibitor of PEK/PERK may also be overexpressed in this brain region. The SCN of the hypothalamus show the highest expression of IMPACT as determined from microarrays (35). We have not quantitated IMPACT or eIF2α(P) in the SCN as a separate region in immunoblots, but immunohistochemistry analyses suggested elevated IMPACT levels in the SCN (data not shown). The SCN are involved in circadian rhythm determination and maintenance. The possibility that the SCN neurons have an even more stringent control over eIF2α phosphorylation is intriguing.

Recent findings concerning activation of GCN2 in the anterior piriform cortex upon feeding a low amino acid diet are not discrepant relative to our results showing high levels of IMPACT in the piriform cortex. Upon close inspection of the anterior piriform cortex, IMPACT was found mainly in interneurons in layer II (data not shown), whereas in animals subjected to a low amino acid diet, only a few pyramidal neurons in the anterior piriform cortex show eIF2α(P) labeling (27). Certainly, co-localization studies will be highly relevant.

YIH1 has been shown to bind to G-actin in yeast. It is not clear what role the interaction between YIH1 and actin plays in yeast cells. However, low actin levels lead to impairment of GCN2 activation, suggesting that the resulting larger pool of free YIH1 yeast cells. However, low actin levels lead to impairment of GCN2 activation, suggesting that the resulting larger pool of free YIH1

pathways associated with learning and memory, there is no information regarding the distribution of ATF4 over different populations of hippocampal neurons. Thus, further understanding of the role played by IMPACT in regulation of the basal and amino acid starvation-induced levels of eIF2α(P) (and consequently, in the levels of ATF4) in specific neuronal cells of the hippocampus may add new insights to the molecular mechanisms involved in long-term potentiation.

Acknowledgment—We thank Dr. Jan van’t Riet for anti-S22 antibodies.

REFERENCES

1. Kaufman, R. J., Scheuner, D., Schroder, M., Shen, X., Lee, K., Liu, C. Y., and Arnold, M. J. (2002) Nature 418, 1425–1428
2. Kozubal, R., Milesi, C., Tremblay, N., and Dignam, J. D. (2003) J. Biol. Chem. 278, 24080–24084
3. Ron, D. (2002) J. Biol. Chem. 277, 4474–4479
4. Foiani, M., Cigan, A. M., Paddon, C. J., Harashima, S., and Hinnebusch, A. G. (2000) Mol. Cell. Biol. 20, 1423–1430
5. Vattem, K. M., and Wek, R. C. (2004) J. Biol. Chem. 279, 11269–11276
6. Yang, R., Wek, R. C., and Wek, R. C. (2000) Mol. Cell. Biol. 20, 6681–6690
7. Zhang, P., McGrath, R. C., Reinitz, J., Olsen, D. S., Lei, L., Gill, S., Wek, S. A., Vattem, K. M., Wek, R. C., Kimball, S. R., Jefferson, L. S., and Cavender, D. R. (2002) Mol. Cell. Biol. 22, 6661–6668
8. De Gracia, D. J. (2001) Neuron 31, 339–349
9. Kim, J. H., Zhu, H., and Kandel, E. R. (2003) Cell 114, 1099–1108
10. Munn, A.-C., Jousse, C., Averous, J., Parry, L., Bruhat, A., Cherasse, Y., and Ito, T. (2005) J. Neurochem. 94, 2913–2923
11. Hagiwara, Y., Hirai, M., Nishiyama, K., Kanazawa, I., Ueda, T., Sakaki, Y., and Ito, T. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 9249–9254
12. Ron, D., and Fisk, J. W. B., and Mathews, M. B., eds) pp. 185–243, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
13.Merchant, N., Wek, R. C., Wek, S. A., and Wek, R. C. (2003) Neuron 39, 655–669
14. Yang, R., Wek, R. C., and Wek, R. C. (2000) Mol. Cell. Biol. 20, 7068–7071
15. Chen, A., Muzzio, I. A., Malleret, G., Bartsch, D., Verbitsky, M., Pavlidis, P., Yonan, A. L., Vronskaya, S., Grody, M. B., Cepeda, I., Gilliam, T. C., and Wek, R. C. (2000) J. Clin. Investig. 110, 1389–1398
16. Yao, S., Sharp, J. W., Ross-Inta, C. M., McDaniel, B. J., Anthony, T. G., Wek, R. C., and Wek, R. C. (2000) J. Biol. Chem. 275, 3203–3216
IMPACT, a Protein Preferentially Expressed in the Mouse Brain, Binds GCN1 and Inhibits GCN2 Activation
Cátia M. Pereira, Evelyn Sattlegger, Hao-Yuan Jiang, Beatriz M. Longo, Carolina B. Jaqueta, Alan G. Hinnebusch, Ronald C. Wek, Luiz E. A. M. Mello and Beatriz A. Castilho

J. Biol. Chem. 2005, 280:28316-28323. doi: 10.1074/jbc.M408571200 originally published online June 2, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M408571200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 36 references, 20 of which can be accessed free at http://www.jbc.org/content/280/31/28316.full.html#ref-list-1