Fibroblast growth factor homologous factors and the islet brain-2 scaffold protein regulate activation of a stress-activated protein kinase* 

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Fibroblast growth factor homologous factors (FHF) form native intracellular complexes with the mitogen-activated protein kinase (MAPK) scaffold protein islet-brain 2 (IB2) in adult brain. FHF binding to IB2 facilitates recruitment of the MAPK p38 (SAPK4), while failing to stimulate binding of JNK, the preferred kinase of the related scaffold IB1 (JIP-1). We now report further biochemical evidence supporting FHFs as regulators of IB2 scaffold activity. Mixed lineage kinase 3 (MLK3) and IB2 synergistically activate p38 but not the MAPKs JNK-1 and p38. Binding of p38 to IB2 is mediated by the carboxyl-terminal half of the scaffold (IB241–436). FHF2 also binds weakly to IB241–436 and can thereby increase p38 interaction with IB241–436. FHF-induced recruitment of p38 to IB2 is accompanied by increased levels of activated p38, and synergistic activation of p38 by MLK3 and IB2 is further enhanced by FHF2. Consistent with a role for FHF as signaling molecules, FHF2 isolated from rat brain is serine/threonine-phosphorylated, and FHF can serve as a substrate for p38 in vitro. These results support the existence of a signaling module in which IB2 scaffolds a MLK3/MKK/p38 kinase cascade. FHFs aid in recruitment of p38 to IB2 and may serve as kinase substrates.

Cellular responses to extracellular signals include activation of mitogen-activated protein kinase (MAPK) pathways that coordinate proliferation, differentiation, apoptosis, and survival. In mammalian cells, the three known branches of MAPKs are extracellular signal-regulated kinases (ERKs), c-Jun amino-terminal kinases (JNKs), and p38 MAPKs, the latter two referred to collectively as stress-activated protein kinases (SAPKs), since they mediate responses both to environmental stresses and to proinflammatory cytokines (1). Activated SAPKs phosphorylate a wide variety of proteins, including transcription factors and cytoskeletal proteins (reviewed in Refs. 2 and 3). MAPKs are typically activated through three-tiered kinase cascades; MAPK kinase kinases (MAPKKKs) activate MAPK kinases (MAPKKs), which in turn activate MAPKs (4, 5). Several different MAPKKKs can activate SAPKs, including TAK1, ASK1, and members of the family of mixed lineage kinases (MLKs). A set of MAPKKs mediates activation of SAPKs, with MKK4 and MKK7 generally activating JNKs and MKK3 and MKK6 generally activating p38.

JNKs are regulators of cell proliferation and apoptosis during development and in response to environmental stress (2). JNK-deficient mice display defects in developmental and excitotoxin-induced neuronal apoptosis (2) and in T-cell function and immune responses (6). Among the four p38 proteins, p38α and p38β play roles in cytokine- and stress-induced actin cytoskeletal reorganization (7–9) as well as in cell differentiation and apoptosis (3). Although no specific biological function has been uncovered for p38δ (also known as SAPK4) (10–12), several observations suggest p38δ to be somewhat different from the prototypical p38α and p38β. In vitro kinase assays have revealed a partially overlapping but distinct substrate specificity for p38δ, including the ability to phosphorylate certain microtubule-associated proteins (13, 14) and eukaryotic elongation factor 2 kinase (15). In addition, some studies suggest that p38δ may be activated by a broader spectrum of MAPKKs, including MKK4 and MKK7 (16).

Scaffold proteins contribute to signaling efficiency and/or specificity of MAP kinase signaling by sequestering MAPK and upstream activating kinases (17). JNK-interacting protein (JIP)-1, also known as islet brain-1 (IB1), is a vertebrate scaffold protein that regulates JNK activity. IB1 was discovered as a JNK-binding protein that also binds MAPK/ERK (MEK) family and the MAPKK MKK7 (18–20). IB1 facilitates JNK activation when overexpressed by transfection together with IB1-interacting upstream kinases (20, 21). Forced overexpression of IB1 or its JNK-binding domain inhibits JNK signaling in many types of cells (18, 19, 22). Endogenous IB1 appears to serve as both a positive and negative regulator of JNK activity. JNK activation in mechanically stressed urethelium is negatively regulated by IB1, as demonstrated by gain- and loss-of-function experiments (23). By contrast, analysis of IB1-deficient mice showed IB1 to be required for JNK activation in hippocampal neurons following exposure to excitotoxins or to anoxic stress (20).

IB2 and its highly similar alternative splice isoform, JIP-2 (Fig. 1A), were discovered through their homology with IB1. IB2 and IB1 share considerable amino acid sequence homology in an N-terminal region encompassing the JNK-binding domain in IB1 and in the C-terminal half including, but not limited to, the Src homology 3 and phosphotyrosine-binding domains (see Fig. 1A). IB2 and JIP-2 interact with MLKs and...
MKK7, and initial studies had identified IB2 and JIP-2 as additional putative scaffolds regulating the JNK pathway (24, 25). In contrast to IB1, however, IB2 interacts in vivo with fibroblast growth factor homologous factors (FHFs) (26). FHFs were initially identified by virtue of their sequence homology to fibroblast growth factors (FGFs) (27–29). However, FHFs are different from FGFs in several ways. FHF proteins are synthesized and localized within native or transfected expressing cells (26, 27), as opposed to the function of FGFs as secreted growth factors acting on extracellular receptors (30). Consistent with FHF intracellular localization, we have shown that FHFs facilitate recruitment of a specific SAPK, p38α, to IB2 in transfected 293T cells (26). These findings have revealed fundamental differences in the signaling complexes assembled by IB2 versus IB1.

Here, taking advantage of the identification of p38α as a potential IB2-regulated kinase, we investigated the role of FHFs in recruitment of p38α to IB2 and subsequent activation of p38α in a tissue culture overexpression system. We show that FHF interaction with IB2 facilitates the association and subsequent activation of p38α. We also show that p38α interacts with the C-terminal half of IB2, which stands in contrast to JNK interaction with the amino-terminal region of IB1 (JIP-1b). Last, we show that FHF2 is serine/threonine-phosphorylated in adult rat brain, consistent with an intracellular function for this IB2-interacting protein.

**MATERIALS AND METHODS**

**Nomenclature and Sequences**—Four murine and corresponding human FHF genes were described by Jeremy Nathans and co-workers (27). Our data base searches identified three of these four FHF genes, which we had originally referred to as FGFs (FGF-11 = FHF3, FGF-12 = FHF1, FGF-13 = FHF2) (28, 29, 31), and FHF4 was given the alternative name FGF-14 (32). Since FHFs so far lack demonstrable FGF-like activity and since we have characterized an intracellular function for these factors, we now refer to these factors solely by the acronym FHF, in order to distinguish them from FGFs. Our previously described FGF-12A is now referred to as FHF1, whereas the alternatively spliced FGF-12B is herein referred to as FHF1B (28).

Alluding to its most prominent sites of expression, we (26) and others (25) have described islet brain-2 (IB2), whereas an alternative splice form of this gene has been described as JIP-2 (24). In the absence of in vivo data implicating IB2 or JIP-2 in regulation of JNK, we have adopted the functionally neutral islet brain nomenclature.

**Mammalian Expression Vectors**—Vectors to express wild type and mutant derivatives of human IB2, murine IB1 (JIP-1B) (20), and human SHP-2 (33) as N-terminal FLAG-tagged proteins have been described as well as vectors to express N-terminal 5× Myc-tagged versions of human FGF1 (GenBank™ accession number B04557) and the long isofrom of FHF2 (34).

pCDNA-T7-MLK3 was constructed by PCR modification of a human MLK3 cDNA template to add the N-terminal T7 tag (MASMTG-GQQMG) prior to cloning into pCDNA3 (Invitrogen). The βisom form of human MKK7 (35) was reverse transcriptase-PCR-amplified from human brain cDNA and cloned into pE81B (36) to attach an N-terminal GST tag. All other kinase expression vectors, including pCDNA-HA-JNK1α1, pCEV29-HA-p65α, pCEFL-HA-p38α, and pCEFL-HA-MLK3, were gifts from R. Silvio Gutkind.

**Antibodies**—Antibodies to epitope tags were as follows: HA tag, mouse monoclonal 12CA5, IgG2b, and Myc tag, mouse monoclonal 9E10, IgG1 (Mt. Sinai School of Medicine Monoclonal Antibody Facility); FLAG tag, mouse monoclonal M2, IgG1 (Sigma); FLAG tag, rabbit polyclonal (Zymed Laboratories Inc.); T7 tag, mouse monoclonal, IgG2b (Novagen); GST tag, mouse monoclonal, IgG1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Rabbits were immunized against peptides corresponding to mouse FHF2 residues 234–244 (GGKSMSSHNEST) synthesized with added N-terminal cysteine for sulfhydryl-mediated directional coupling to carrier protein (Research Genetics). Antibodies were affinity-purified with corresponding peptides immobilized on activated agarose ( Pierce). FHF2 antibodies recognized FHF2, but not FHF-1, in lysates from
expressed in 293T cells (Fig. 2A) [37]. Cells were transfected with epitope-tagged protein expression vectors, and aliquots of subsequent cell lysates were analyzed by Western blotting in kinase assays. A, cells were co-transfected with FLAG-tagged IB2 and HA-tagged p38α and stimulated with H2O2, and lysates were electrophoresed, blotted, and probed with anti-HA antibodies or with antibodies against phospho-p38 (p-p38). Anti-HA immunoprecipitates were added to kinase assay reactions containing GST-ATF-2 substrate and (γ-32P)ATP, which were subsequently expressed in vitro and phosphorylated. [32P]Incorporation into ATF-2 was visualized by autoradiography. B, cells were co-transfected with FLAG-tagged IB2 and HA-tagged p38α and stimulated with anisomycin, and lysates were electrophoresed, blotted, and probed with anti-HA antibodies or with antibodies against p-p38. C, lysates from cells co-transfected with HA-tagged p38α together with either GST-tagged MKK7 or empty vector were immunoprecipitated with anti-HA antibodies and subjected to kinase assays as described above. Products were subsequently electrophoresed and blotted. [32P]ATF-2 levels were visualized by autoradiography and analyzed using PhosphorImager imaging and ImageQuant software and recalculated as activity relative to the activity of p38α in the absence of MKK7. The same blot was probed with anti-HA to detect HA-p38α proteins.

RESULTS

p38α Is an IB2-regulated Kinase—IB2 can complex with p38α but not other MAPKs, including JNK1, in transfected 293T cells [26]. These findings suggested that IB2 may regulate p38α kinase activity. As a means to survey the putative involvement of IB2 in regulation of p38α, we tested whether IB2 was capable of stimulating or inhibiting activation of p38α in 293T cells. Basal activity of p38α, detected by the presence of phospho-p38α, was found to be weakly activated in some experiments by coexpression of IB2 (Fig. 2A). Additionally, IB2 increased activation of p38α induced by peroxide stress (Fig. 2A). By contrast, activation of p38α by anisomycin stress was inhibited by IB2 expression (Fig. 2B), similar to findings reported for effects of IB1 (JIP-1) on JNK activation [20]. These findings demonstrate that IB2 can positively or negatively modulate signaling pathways leading to p38α activation.

IB2 binds to the MAPKKK MLK3 and related members of the family of mixed lineage kinases but not to several other MAPKKks (24) and binds to the MAPKKK MKK7 but not to other MKKs (24, 25).2 Hence, IB2 is envisaged to scaffold a

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MAPK pathway, enlisting MLKs and MKK7 as upstream kinases of p38. However, the published data regarding the ability of MKK7 to activate p38 is conflicting (10, 16). To address this issue, we tested directly the ability of MKK7 to activate p38 in vitro (Fig. 3, A). Activation of JNK by increasing amounts of MLK3 was not enhanced by the inclusion of IB2, whereas heterotrophic FHF1/IB2/p38 complexes were assayed by anti-HA Western blot after anti-FLAG immunoprecipitation (Fig. 4, B and D), in contrast to the above findings, a IB2 fragment spanning regions II and III (IB2529–529) severely impaired heterotrimeric complex formation as anticipated (Fig. 4C). Consistent with the above findings, a IB2 fragment spanning regions II and III (IB2514–436) retained the ability to interact with p388 (Fig. 4B). These data define the IB2 C-terminal homology regions II and III as bearing the determinants for p38 binding.

A Weak FHF-binding Site on IB2 Involved in Formation of FHF/IB2/p388 Complexes—FHF2, IB2, and p388 assemble into a heterotrimeric complex when overexpressed in 293T cells, and deletion of the major FHF-binding domain (residues 212–471) nearly abolishes the ability of IB2 to assemble such a complex (26) (Fig. 4C). IB2 deletion of part of the p388-binding domain (IB2459–529) severely impaired heterotrimeric complex formation as anticipated (Fig. 4C). Surprisingly, IB2514–436 was able to assemble into heterotrimeric complexes with FHF8 and p388 (Fig. 4C). This finding suggested that an additional binding site for FHF might be present within IB2514–436. We screened for additional FHF binding regions in IB2, overexpressing tagged proteins in cotransfected 293T cells. As reported before, wild type IB2 binds FHF2 strongly, whereas IB2514–471 (missing the major FHF-binding domain) did not bind at all (Fig. 5). The C-terminal segment of IB2 resulting from further truncation of the amino terminus (IB2514–436) bound FHF weakly (Fig. 5). This finding,
together with previous mapping of FHF/IB2 interaction domains (26) (summarized in Fig. 1B), suggested that the C-terminal half of IB2 harbors a weak binding site for FHF as well as p38/H9254 and that the weak FHF-binding site is masked by a more amino-terminal region of IB2. Consistent with the presence of both a weak FHF binding site and a p38/H9254 binding site in the C-terminal half of IB2, interaction between IB2\(_{21-436}\) and p38 was modestly potentiated by cotransfection of FHF2 (Fig. 4D). These results suggest that the weak FHF-binding domain in the C-terminal half of IB2 contributes to FHF-induced recruitment of p38 to IB2.

**Fig. 4.** Carboxyl-terminal half of IB2 mediates p38\(\delta\) binding. A, IB2-p38\(\delta\) complex formation is FHF-independent at high IB2 concentration. 293T cells were transfected with plasmids expressing HA-p38\(\delta\) (0.05 \(\mu\)g), FLAG-IB2 (0.5 or 1.5 \(\mu\)g), and Myc-FHF2 (0 or 2.5 \(\mu\)g), lysates were immunoprecipitated with anti-FLAG, and the blot was probed with anti-HA to detect IB2-p38\(\delta\) complexes. Lysates were analyzed for expression of all proteins by appropriate blots, as indicated. B, the C-terminal half of IB2 is essential for p38\(\delta\) interaction. 293T cells were transfected with plasmids expressing HA-p38\(\delta\) and FLAG-tagged IB2 (wild-type or mutants; Fig. 1B) at high IB2 plasmid concentrations (1.5 \(\mu\)g). Anti-FLAG immunoprecipitation and Western blot probing with anti-HA assayed for IB2-p38\(\delta\) complexes, and Western blots of total cell lysates were probed to detect p38 and IB2 proteins. C, heterotrimeric FHF/IB2-p38\(\delta\) complex detection. 293T cells were transfected with expression plasmids for HA-p38\(\delta\), Myc-tagged FHF2 or FGF1, and FLAG-tagged IB2 proteins (wild-type or mutant; Fig. 1B). Lysates were analyzed for expression of all proteins with appropriate antibodies, as indicated, and also used in anti-Myc immunoprecipitation for Western blot probing with anti-HA to detect FHF/IB2/p38\(\delta\) heterotrimeric complexes. D, potentiation of IB2\(_{21-436}\) p38\(\delta\) complex formation by FHF2. Lysates from 293T cells cotransfected with plasmids for HA-p38\(\delta\) and FLAG-IB2\(_{21-436}\) in the presence or absence of Myc-FHF2 were immunoprecipitated with anti-FLAG, and the blot was probed with anti-HA to detect IB2\(_{21-436}\)-p38\(\delta\) complexes. Samples of total lysates were used to detect expression of all proteins.
FHF Promotes p38\(\delta\) Activation through Recruitment of p38\(\delta\) to IB2—To determine whether FHF-induced recruitment of p38\(\delta\) to IB2 favors p38\(\delta\) activation, we expressed tagged versions of these signaling proteins in 293T cells at levels sensitive to FHF induction of scaffold-kinase complexes. Subsequently, p38\(\delta\) kinase activity was assayed by Western blot using antibodies against phospho-p38 or in kinase assays using ATF-2 as in vitro substrate. When cells were transfected with p38\(\delta\) and IB2, a low basal level of p38\(\delta\) activity was observed. In the presence of increasing concentrations of FHF2, p38\(\delta\) phosphorylation and kinase activity increased commensurately (Fig. 6A).

We next tested the separate and combined effects of FHF2, MLK3, and IB2 on activation of p38\(\delta\). As shown in Fig. 6B, expression of IB2 by itself did not stimulate p38\(\delta\) activity, and expression of MLK3 alone stimulated p38\(\delta\) activity only 2.2-fold. By contrast, coexpression of MLK3 with IB2 stimulated p38\(\delta\) activity 19-fold. In the absence of IB2, FHF was unable to stimulate p38\(\delta\) activity, nor could it potentiate the weak activation of p38\(\delta\) by MLK3. By contrast, FHF2 augmented the IB2-mediated activation of p38\(\delta\) by MLK3 (Fig. 6B). Reflecting the binding specificity of IB2 for p38\(\delta\), IB2 could not potentiate MLK3-mediated activation of p38\(\delta\) (Fig. 6B). These results demonstrate that FHF-mediated recruitment of p38\(\delta\) to IB2 enhances the signaling efficiency of the IB2 module.

FHF Phosphorylation—For each FHF gene, mRNAs generated by alternative splicing of transcripts encode variant isoforms differing in N-terminal sequence (28, 32, 38); for FHF2, the predicted isoforms are 21.5 and 27.5 kDa (GenBank\textsuperscript{TM} accession numbers NM_033642 and NM_010200, respectively). We investigated the expression and sizes of FHF2 isoforms in extract of rat cerebral cortex by Western blot using antibodies raised against an epitope in the common C terminus of FHF2 variants.

FHF2 species corresponding to both predicted isoforms were detected (Fig. 7A, lane 1). However, when the extracts were subjected to immunoprecipitation with the same antibody prior to Western blot, the 28-kDa species was significantly underrepresented (Fig. 7A, lane 2). Treatment of immunoprecipitates with phosphatase PP2a restored detection of the 28-kDa species (Fig. 7A, lane 3), and this effect was blocked when the phosphatase inhibitor okadaic acid was included in the treatment (Fig. 7A, lane 4). These findings demonstrate that the longer FHF2 isoform is phosphorylated on serine or threonine residues, rendering its immunoprecipitation and detection by antibodies inefficient. FHF2 is most likely phosphorylated within the C-terminal epitope, which served as immunogen,
tates. Washed immunoprecipitates were left untreated (lane 3) treated with PP2a (lane 2) and left untreated or induced with H2O2. HA-p38 was probed with anti-HA to detect HA-p38.

Additional FHF2 species ranging from 30,000 to 34,000 in molecular weight species (Fig. 7, lanes 1-2, total extract; lanes 2-4, anti-FHF2 immunoprecipitates). A prominent band at 20 kDa is indicated as well as the lower and upper bands of a 28–34-kDa ladder. The 28-kDa band is also indicated in lane 3. B, FHF1 is a substrate for p38 in vitro. 293T cells were transfected with plasmids expressing HA-p38 and left untreated or induced with H2O2. HA-p38 activity in extracts was assayed in kinase assays containing bacterially expressed FHF1 as a substrate and [γ-32P]ATP, which were subsequently electrophoresed and blotted. 32P incorporation into FHF1 was visualized by autoradiography. The same blot was probed with anti-HA to detect HA-p38 proteins.

Since FHF associates with the IB2 scaffold and is phosphorylated in vivo, we considered the possibility that FHFs are substrates of the scaffolded kinase cascade. To preliminarily address this question, HA-tagged p38 is from untreated or peroxide-treated cells was immunoprecipitated and incubated in vitro with recombinant, bacterially expressed FHF1. Phosphorylation of FHF1 was detected after incubation with peroxide-activated p38 but not with inactive p38 (Fig. 7B), demonstrating that FHF-1 can serve as a p38 substrate in vitro. Phosphorylation of FHFs in vivo and in vitro provides further evidence for intracellular FHF function and suggests that this function may be regulated by the kinase complex associated with IB2.

**DISCUSSION**

A p38 Kinase Cascade Coordinated by IB2—Insol brain proteins are kinase scaffolds and mediate MAPK activation through MLKs and MKK7 in transfected cells. JNKs have been identified as the exclusive targets of IB1 regulation (24). We identified IB2 as an FHF-binding protein and showed it to bind p38 in contrast to JNKs or other MAPKs (26). We have now shown that FHF-induced binding of IB2 to p38 is associated with activation of p38 and that IB2 can facilitate activation of p38 by MLK3. We thereby suggest p38 to be an IB2-regulated MAPK, making IB2 the first vertebrate scaffold protein found to bear specificity for a member of the p38 subfamily of MAPKs. This model is also consistent with the ability of MKK7 to activate p38 (Fig. 1) (16). The coupling of MLK3 and MKK7 to JNK and p38 by IB1 and IB2, respectively, extends to vertebrates a principle of kinase target specificity first appreciated in yeast, wherein the MAPKKK STE11 is channeled toward FUS3 or HOG1 MAPK in response to different cell stimuli acting through different kinase scaffolds (39).

Previous studies from Yasuda et al. (24) reported weak binding of the N-terminal IB2 variant JIP-2 to JNKs in transfected COS cells, and IB2 potentiated MLK3-induced JNK activation in these cells. By contrast, we find that IB2 fails to bind JNK1 in 293T cells (26). Moreover, in many experiments in 293T cells with varying levels of IB2, JNK, and upstream kinase, JNK activity was not significantly enhanced in the presence of IB2 (Fig. 2). IB2, instead, could bind to p38 and facilitate p38 activation (see Ref. 26; this paper). Furthermore, the amino-terminal variant JIP-2 binds to p38 as effectively as does IB2 in 293T cells.

We suggest that the differences between our findings and those of Yasuda et al. (24) may result from different scaffold/kinase interactions in different cellular contexts.

The IB2 scaffold and FHFs are principally expressed in the nervous system (25, 28) along with several known or putative scaffold-associated kinases (i.e. DLK, MLK3, JNK, and p38) (2, 12, 40). The assignment of specificity of IB2 scaffold toward the JNK or p38 kinases is based so far on evidence obtained in overexpression assays in nonneuronal tissue culture cells. Whereas interaction of endogenous IB2 with FHFs in the brain has been validated (26), identification of the actual MAPKs assembled by IB2 in vivo still await confirmation.

Kinase Interactions with Islet Brain Scaffolds—A short amino-terminal segment of IB1 (JIP-1B; residues 152–162) has been described as its JNK-binding domain (18, 20). Although this segment bears 55% sequence identity to a corresponding segment of IB2 (residues 106–116), we failed to detect direct binding of p38 to this homology region in IB2. The p38-binding domain of IB2 includes residues C-terminal to the major FHF-binding domain, which are related to IB1 (homology regions II and III). In other words, despite well aligned regions of sequence homology, the MAPK-binding segments of IB2 and IB1 appear unrelated in position and sequence. De-

3 J. Schoorlemmer, unpublished data.
spite these unanticipated findings, it is still possible that IB1 and IB2 fold to create similar MAPK binding surfaces consisting of N-terminal region I and more C-terminal elements; within this common fold, IB2 and IB1 could differ in the residues that primarily contribute to stabilizing interactions with their respective kinases. We also note that the ability of IB2 to homoooligomerize or heterooligomerize with IB1 (JIP-1) (24) raises the possibility that these scaffolds may coordinately activate both JNK and p38.

Islet brain kinase scaffolds were initially modeled as bearing multiple domains, each able to bind all kinases in a cascade simultaneously (39). However, islet brain scaffolds may bind kinases sequentially, since JNK binding to IB1 decreases the affinity of DLK or MLK3 to the scaffold by an unknown mechanism, resulting in their activation (21). Preliminary studies have identified the C-terminal half of IB2 (JIP-2), containing homology domains II and III, as the binding region for MLK3, MKK7 (24), and p386 (this study). Our preliminary analysis in 293T cells, using IB2 mutants lacking either domain II or III, has failed to assign independent separable binding sites for each of these kinases; each of these mutants (IB2Δ459–529 and IB2Δ514–791) displayed reduced binding to both p386 (Fig. 4, B and C) and MLK3.3 In other words, homology domains II and III may contribute to a binding surface for multiple kinases. Our findings leave open the possibility that IB2 scaffold binding sites for different kinases overlap and preclude simultaneous occupancy. If so, a signaling unit containing multiple kinases may be assembled through oligomerization of IB2 chains, each harboring different kinases.

FHF's Function through Interactions with Intracellular Proteins—Although FHFs bear substantial sequence homology with the core region of FGFs, FHFs complex with IB2 to recruit p386, pointing toward an intracellular role in MAPK signaling (26). The relationship of FHFs to protein phosphorylation is now further supported by additional findings: 1) FHFs positively regulates IB2 function, facilitating the ability of IB2 to mediate the activation of p386; 2) FHFs is a phosphoprotein in vivo.

Analysis of truncated IB2 proteins has defined the C-terminal 360 residues of IB2 as possessing the binding site for p386 (Fig. 4, B and C), along with a minor binding site for FHF (Fig. 4D). Hence, IB2Δ31–436 can form heterotricomplexes with FHF and p386, and FHF can potentiates IB2Δ459–891p386 interaction. More upstream IB2 sequences, within a region bearing no sequence homology to IB1, are required for strong interaction with FHF. Potentially, FHFs may interact with both major and minor FH-binding domains on IB2 to promote a stronger affinity of the complex toward p386.

Whereas IB2 is expressed in apparently all neurons of the central and peripheral nervous systems,4 FHFs expression is restricted to subsets of neurons (27, 28, 32). Regulation of FHFs expression may be a direct stimulus for MAPK activation on IB2. Alternatively, FHFs may serve as cofactor for MAPK recruitment to IB2, allowing for kinase activation by other stimuli in FHF-positive neurons. Furthermore, FHFs phosphorylation in brain and by p386 in vitro opens to speculation the possibility that FHFs is a target of IB2-scaffolded kinases.

Potential Function of the IB2 Signaling Module—The JNK scaffold IB1 is essential for stress-induced apoptosis in hippocampal neurons and has been suggested to connect kinesin motors for microtubular transport to vesicular cargoes. By contrast, the biological functions of IB2 are currently unknown. Here we speculate regarding IB2 function, based upon current knowledge of IB1 function together with our findings.

Kainate stress results in JNK- and c-Jun-mediated neuronal apoptosis in the brain that is dependent upon IB1. IB1 is also required for activation of JNK and subsequent apoptosis induced by anoxia combined with glucose deprivation in brain and hippocampal neurons, as may happen during ischemia (2, 41). By contrast, IB1 is not required for UV light- or anisomycin-induced JNK activation in hippocampal neurons (41), indicating that specific SAPK pathways are engaged by different stress stimuli. We speculate that IB2 may play a role in neuronal pathophysiological stress responses in reaction to specific stimuli that might not engage IB1. If so, IB2 and FHFs may direct signaling toward a different output as compared with IB1 through regulation of p386.

Trafficking of IB1 to growth cones in differentiating neurons depends upon IB1 interaction with the cargo-binding domain of the light chain subunit of kinesin I, a major motor protein for axonal transport (42). Since IB1 also interacts with the intracellular domains of transmembrane proteins in vitro (43, 44), it has been inferred that the IB1 scaffold might connect kinesin motors to vesicular cargoes (42, 45, 46). This might illustrate a more generalized function for kinase scaffolds, since a role of the structurally unrelated JNK-scaffold JIP-3 in axonal transport has been firmly established (47, 48). Directed transport of IB1 may serve to deliver preformed signaling complexes to defined intracellular locations. Alternatively, IB1-associated kinases may regulate the transport process itself or association of the scaffold to vesicular cargo (42).

Consistent with kinesin-dependent trafficking of IB2 to a peripheral location in neuronal cells (42) and conservation of the amino acids required for kinesin binding in islet brain scaffolds, IB2 can be assumed to be transported along microtubules by mechanisms similar to IB1. However, IB2 may not function identical to IB1. The localization of IB2 is overlapping with but distinct from IB1 in pancreatic β cells (24) and in the axons of cerebral cortical neurons. Moreover, the affinity of IB2 for at least some transmembrane proteins in putative vesicular cargoes is different from IB1 (49). Finally, IB2 possesses the unique property of regulating p386 in conjunction with FHFs, potentially allowing regulation of distinct targets such as microtubule-associated proteins involved in transport (43, 44). Altogether, IB2 might add a regulatory function of intracellular traffic and, in conjunction with FHFs, to localize SAPK signaling as part of the pleiotropic cellular responses to pathophysiological stress.

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