FEBS Letters 564 (2004) 14–18 FEBS 28242

FRS2 family docking proteins with overlapping roles in activation of MAP kinase have distinct spatial-temporal patterns of expression of their transcripts

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Received 18 February 2004; accepted 24 February 2004

First published online 1 April 2004

Edited by Veli-Pekka Lehto

Abstract FRS2α and FRS2β, two members of the FRS2 family of docking proteins, become tyrosine phosphorylated in response to fibroblast growth factor (FGF) or nerve growth factor (NGF) stimulation. Tyrosine phosphorylated FRS2α serves as a platform for the recruitment of multiple signaling proteins for activation of the Ras-mitogen-activated protein (MAP) kinase signaling cascade. We report that *Frs2α* and *Frs2β* have distinct spatio-temporal expression patterns in mouse embryos. We further show that FRS2β can compensate for the loss of FRS2α for activation of MAP kinase when expressed in fibroblasts from *Frs2α*$^{-/-}$ mouse embryos. We propose that the FRS2 family proteins have distinct roles in vivo through activation of common signaling proteins including MAP kinase.

Key words: Docking protein; FRS2; Fibroblast growth factor; Neurotrophin; Mitogen-activated protein kinase; In situ hybridization

1. Introduction

Docking proteins FRS2α and FRS2β play important roles in mediating the intracellular signals induced by fibroblast growth factors (FGFs) and members of the nerve growth factor (NGF) and glial cell-derived neurotrophic factor families of neurotrophins [1–5]. FRS2α acts as a platform for direct and indirect recruitment of a variety of signaling protein complexes to regulate a diverse array of cellular responses. We created a targeted deletion of the *Frs2α* gene in mice and showed that FRS2α deficiency causes embryonic lethality at embryonic day (E)7–7.5 due to a defect in FGF4 signaling for development of trophoblast tissues, underscoring the critical role of FRS2α in early embryogenesis ([6] and unpublished results). FGFs and neurotrophins are involved in development of multiple tissues including those of the nervous system, and it is possible that FRS2α also plays an important role in the development of these tissues [7,8].

We used fibroblasts from *Frs2α*$^{-/-}$ embryos to show that FRS2α plays an important role in mediating multiple FGF-induced cellular responses including stimulation of mitogen-activated protein (MAP) kinase and phosphatidylinositol (PI)-3 kinase [6]. In addition to the central role of MAP kinase in FGF-induced cell proliferation and differentiation, MAP kinase plays a key role in a negative feedback mechanism in which MAP kinase phosphorylates multiple threonine residues in FRS2α [9]. The mechanisms of intracellular signaling through FRS2β remain unclear.

In the present study, we examined the expression patterns of *Frs2α* and *Frs2β* in mouse embryos by in situ hybridization. We also examined the possibility that FRS2α and FRS2β have redundant roles in signaling via FGF receptors (FGFRs) through ectopic expression of FRS2β in *Frs2α*$^{-/-}$ mouse embryonic fibroblasts (MEFs) that are also deficient in FRS2β.

2. Materials and methods

2.1. Cloning of FRS2β cDNA and in situ hybridization

Human *FRS2β* full-length cDNA was cloned by screening the human brain cDNA library (Clontech) with a ~1-kb partial fragment of human *FRS2β* cDNA from an EST clone. Full-length cDNA of mouse *Frs2β* was cloned by screening the mouse brain cDNA library (Clontech) with a ~500-bp fragment of human *FRS2β* cDNA. For whole-mount RNA in situ hybridization experiments, the mouse *Frs2α* cDNA fragment linearized with *Hpa*I or the mouse *Frs2β* full-length cDNA linearized with *Bam*HI were used as specific probes. For section RNA in situ hybridization experiments, mouse *Frs2α* or *Frs2β* cDNAs were linearized with *Not*I or *Bam*HI, respectively, and then used as specific probes. Antisense riboprobes and sense riboprobes were synthesized with digoxigenin (DIG)-coupled uridine triphosphate (UTP) (Boehringer-Mannheim). Whole-mount RNA in situ hybridization was performed essentially as described [10]. Section RNA in situ hybridization was performed essentially as described [11]. BM purple was used as a substrate for alkaline-phosphatase-coupled anti-DIG antibodies (Boehringer-Mannheim). After hybridization, the sections were counterstained with Nuclear Fast Red (Vector Lab.).

2.2. Cell culture and generation of MEFs expressing FRS2β

MEFs derived from *Frs2α*$^{-/-}$ embryos and human embryonic kidney (HEK) 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FBS) from Gibco BRL as previously described [6]. *FRS2β* cDNA with a Myc tag attached to the

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Abbreviations: FGF, fibroblast growth factor; NGF, nerve growth factor; DRG, dorsal root ganglia; VZ, ventricular zone; MEF, mouse embryonic fibroblast

0014-5793/04/$30.00
doi:10.1016/S0014-5793(04)00287-X

C-terminus of *FRS2β* was cloned into the mammalian expression vector pBABE and a high-titer virus stock was produced as described previously [2]. *Frs2α*$^{-/-}$ MEFs were infected with retrovirus containing pBABE alone or *FRS2β* cDNA that was cloned into pBABE and selected for 2 weeks in medium supplemented with puromycin (1 μg/ml). Pools of selected cultures were used for experiments. HEK 293 cells were transfected using lipofectamine (Invitrogen) according to the manufacturer's protocol.

2.3. Immunoprecipitation and immunoblot analysis

Cells were starved overnight in 0.5% serum and stimulated with FGF1 (100 ng/ml) together with heparin (5 μg/ml) for 5 min at 37°C. Subsequently, the cells were lysed and subjected to immunoprecipitation followed by immunoblotting as described [12]. Anti-FRS2, anti-Shp2, anti-phosphotyrosine, anti-Grb2, anti-phospho-specific Erk and anti-Erk antibodies were described previously [12]. Anti-Myc tag antibody, clone 9E10, was purchased form Upstate Biotechnology.

3. Results and discussion

3.1. Expression of Frs2α and Frs2β in mouse embryos

The patterns of expression of *Frs2α* and *Frs2β* were compared by in situ hybridization of mouse embryos at different stages of development. Whole-mount in situ hybridization of E8.5 embryos with antisense probe for *Frs2α* revealed high levels of ubiquitous expression of *Frs2α* transcript in the embryo (Fig. 1A), whereas levels of expression of *Frs2β* were not above background in any areas of the embryos (Fig. 1C). Sense probes for *Frs2α* and *Frs2β* were used as controls for specificity of in situ hybridization experiments (Fig. 1B and data not shown). We also performed in situ hybridization of *Frs2α* on frozen sagittal sections of embryos at E9.5 and E11.5. We found that at E9.5, *Frs2α* is expressed ubiquitously in most developing tissues (Fig. 1D), whereas *Frs2β* expression was negligible (Fig. 1F). Sense probes for *Frs2α* and *Frs2β* were used as controls for specificity of in situ hybridization experiments (Fig. 1E and data not shown).

As shown in Fig. 1G, at E11.5 *Frs2α* transcripts were present in many developing tissues, including the first and second branchial arches (such as tongue and jaw), neuroepithelium of the brain (arrowheads), hepatic parenchyma, midgut, and precartilage primordium of the vertebrae and hindlimb (Fig. 1G). We also observed cells that only showed

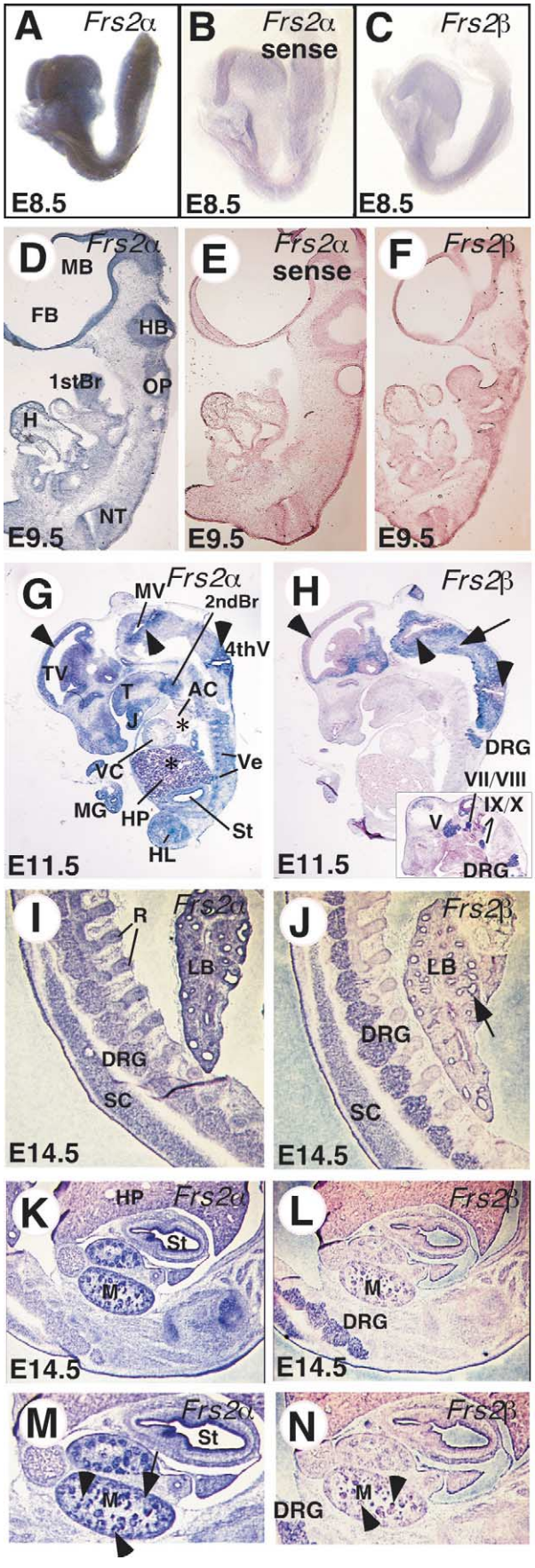

Fig. 1. Expression of *Frs2α* and *Frs2β* in mouse embryos. Whole-mount in situ hybridization with *Frs2α* antisense probe of mouse embryos (A–C). In situ hybridization for frozen sagittal sections of embryos (D–N). Specific binding of the antisense probes is visible as blue or violet staining (A–N). The same sections were counterstained with Nuclear Fast Red to visualize tissue structures (D–N). Embryonic stages and antisense probes used for staining are indicated in each image. We used adjacent sections in D, E and F, G and H, I and J, and K and L, and M and N. Arrowheads in G and H indicate VZ of the neuroepithelium. An arrow in J indicates lung epithelium. An arrow in M indicates metanephric mesenchyme. Arrowheads in M and N indicate tubuli or s- or comma-shaped pretubular structure of the metanephric kidney. FB, forebrain; MB, midbrain; HB, hindbrain; OP, otic pit; 1st Br, first branchial arch; H, heart; NT, neural tube; TV, telencephalic vesicle; MV, mesencephalic vesicle; 4th V, fourth ventricle; T, tongue; J, jaw; 2nd Br, second branchial arch; AC, atrial chamber; VC, ventricular chamber; Ve, precartilage primordium of vertebrae; HP, hepatic parenchyma; MG, midgut; St, stomach; HL, hindlimb; V, trigeminal ganglion; VII/VIII, facial (VII)–acoustic (VIII) ganglion complex; IX/X, glossopharyngeal–vagus ganglion complex; R, cartilage primordium of ribs; LB, lung bud; SC, spinal cord; M, metanephric kidney. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the pink counterstaining in the chambers of the heart and venous sinusoids lining the gap of loose hepatic parenchyma (Fig. 1G, *). These cells appeared to be primitive nucleated red blood cells and did not express *Frs2α* at a detectable level. Strong expression of *Frs2β* was detected predominantly in the neuroepithelium at E11.5 (Fig. 1H, arrow). *Frs2β* was also expressed in dorsal root ganglia (DRG) and in cranial ganglia such as the trigeminal (V) ganglia (Fig. 1H, inset).

In situ hybridization of embryos at E14.5 revealed ubiquitous expression of *Frs2α*. *Frs2α* was strongly expressed in the lung bud, cartilage primordium of ribs, DRG and spinal cord (Fig. 1I). *Frs2α* was also expressed strongly in the metanephric mesencyme, tubuli and s- or comma-shaped pretubular structure of the metanephric kidney and stomach epithelium (Fig. 1K, M). At E14.5, *Frs2β* was expressed at high levels in neural tissues in DRG and spinal cord and in several tissues such as lung epithelium (Fig. 1J) and tubuli and s- or comma-shaped pretubular structure of the metanephric kidney (Fig. 1L, N).

These results indicate that *Frs2α* is expressed in most developing tissues, whereas *Frs2β* is expressed predominantly in the developing neuroepithelium.

3.2. *Distinct patterns of expression of* Frs2α *and* Frs2β *in embryonic brain*

To further analyze the expression of *Frs2α* and *Frs2β* in neural tissues, we compared the patterns of expression of these genes in coronal sections of the heads of E14.5 embryos. We observed strong expression of both transcripts in the brain and trigeminal ganglia (V) (Fig. 2A–D). *Frs2α* was also expressed in other tissues such as cartilage primordium (Fig. 2C, arrowhead). The layer adjacent to a ventricle is ventricular zone (VZ) and is composed of germinal neuroepithelium containing immature neuroblasts and glioblasts. Once cells in this region stop dividing, they migrate away from the ventricle and begin to differentiate [13]. At E14.5, *Frs2α* but not *Frs2β* was expressed strongly in the VZ surrounding the lateral ventricles and the mesencephalic vesicle (Fig. 2A–D, *). The reciprocal pattern of expression of both genes was also seen in the neuroepithelium at E11.5 (Fig. 1G, H, arrowheads). We next analyzed expression of *Frs2α* and *Frs2β* in coronal sections of brain of E18.5 embryos. Both *Frs2α* and *Frs2β* were expressed in the cortical plate and cortical subplate in the cingulate and parietal cortices and caudate putamen (Fig. 2E–H). Strong expression of *Frs2β* was observed in the lateral septal nucleus, thalamus and hypothalamus, whereas *Frs2α* was expressed at low levels in these areas of the brain (Fig. 2E–J). Finally, both genes were expressed in the CA1, CA2, and CA3 regions of the developing hippocampus and in dentate gyrus (Fig. 2I, J).

3.3. *FRS2β rescues a defect in MAP kinase stimulation in* $Frs2\alpha^{-/-}$ *cells*

We next examined whether FRS2β mediates cellular responses previously shown to be mediated by FRS2α in response to FGF stimulation. Transient coexpression experiments in HEK 293 cells were performed with vectors expressing FLAG-tagged FRS2α or FRS2β and vectors expressing FGFR1 or NGF receptor (NGFR). Both FRS2α and FRS2β were tyrosine phosphorylated and formed complexes with Grb2 in response to FGF or NGF (Fig. 3A). Both FRS2α and FRS2β bound activated and tyrosine phosphorylated FGFR and NGFR (Fig. 3A). As previously shown, FRS2α but not FRS2β was phosphorylated on threonine residues resulting in reduced electrophoretic mobility (Fig. 3B). We also expressed Myc-tagged FRS2β in MEFs derived from $Frs2\alpha^{-/-}$ embryos. Lysates from unstimulated or FGF-stimulated cells were subjected to immunoprecipitation with anti-Myc antibodies followed by immunoblotting with anti-FRS2, anti-phosphotyrosine, anti-Grb2, or anti-Shp2 antibodies. As shown in Fig. 3C and D, FGF stimulation induced tyrosine

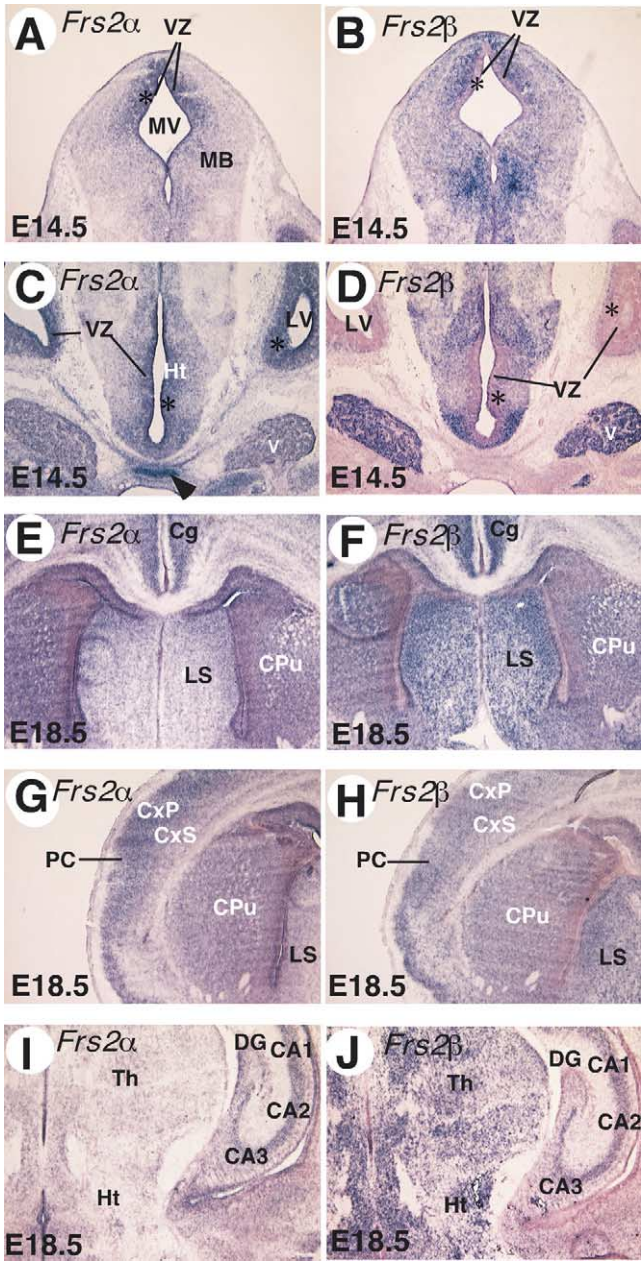

Fig. 2. Expression of *Frs2α* and *Frs2β* in heads of E14.5 and E18.5 embryos. In situ hybridization for frozen coronal sections of heads. Embryonic stages and antisense probes used for staining are indicated in each image. Images in A and B, C and D, E and F, and G and H are from single sections, respectively. In the head of embryos at E18.5, compared with the midline, sections in E–H are anterior, and sections in I and J are posterior. LV, lateral ventricle; Ht, hypothalamus; Cg, cingulate cortex; LS, lateral septal nucleus; CPu, caudate putamen; CxP, cortical plate; CxS, cortical subplate; PC, parietal cortex; Th, thalamus; DG, denta gyrus.

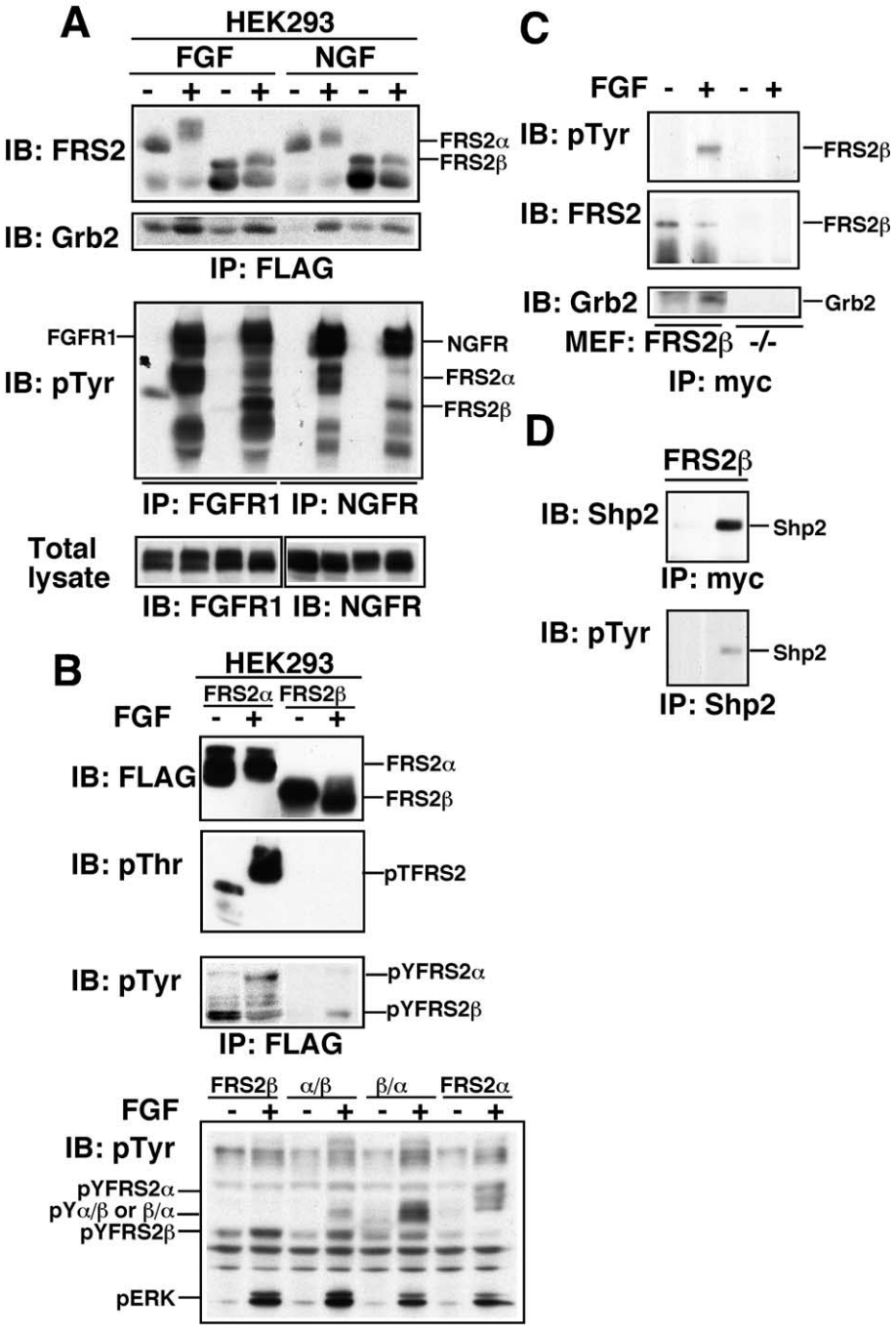

Fig. 3. FGF and NGF induce tyrosine phosphorylation of FRS2β in transfected cells. A: HEK 293 cells were cotransfected with vectors expressing FRS2α-FLAG or FRS2β-FLAG and vectors expressing FGFR1 or NGFR. Lysates from unstimulated or stimulated cells were subjected to immunoprecipitation with anti-FLAG antibodies, anti-receptor antibodies followed by immunoblotting with anti-FRS2, anti-Grb2 and anti-phosphotyrosine antibodies (p-Tyr). B: HEK 293 cells were cotransfected with vectors expressing FRS2α-FLAG or FRS2β-FLAG (top panel) and vector expressing FGFR1. Lysates from unstimulated or stimulated cells were immunoprecipitated with anti-FLAG antibodies and immunoblotted with antibodies directed against a consensus MAP kinase phosphorylation site, (designated p-Thr), anti-phosphotyrosine antibodies, or anti-FLAG antibodies. Lysates from HEK 293 cells that were transfected with expression vectors that direct the synthesis of FRS2α, FRS2β, the chimeric α/β FRS2 or the chimeric β/α FRS2 were subjected to immunoblotting with anti-phosphotyrosine antibodies (bottom panel). C, D: Lysates from unstimulated or FGF1-stimulated *Frs2α*$^{-/-}$ MEFs or MEFs expressing FRS2β-Myc were subjected to immunoprecipitation with anti-Myc or anti-Shp2 antibodies followed by immunoblotting with the indicated antibodies.

phosphorylation of FRS2β as well as complex formation between FRS2β and Grb2 and Shp2. We previously showed that FGF-induced stimulation of MAP kinase was compromised in *Frs2α*$^{-/-}$ cells. We next examined whether ectopic expression of FRS2β in FRS2-deficient MEFs rescues the MAP kinase response in response to FGF. *Frs2α*$^{-/-}$ MEFs expressing FRS2β or FRS2α or vector-transfected *Frs2α*$^{-/-}$ MEFs were stimulated with FGF, and activation of MAP kinase was monitored by immunoblotting of lysates from unstimulated or stimulated cells with anti-phospho-Erk antibodies. As re-

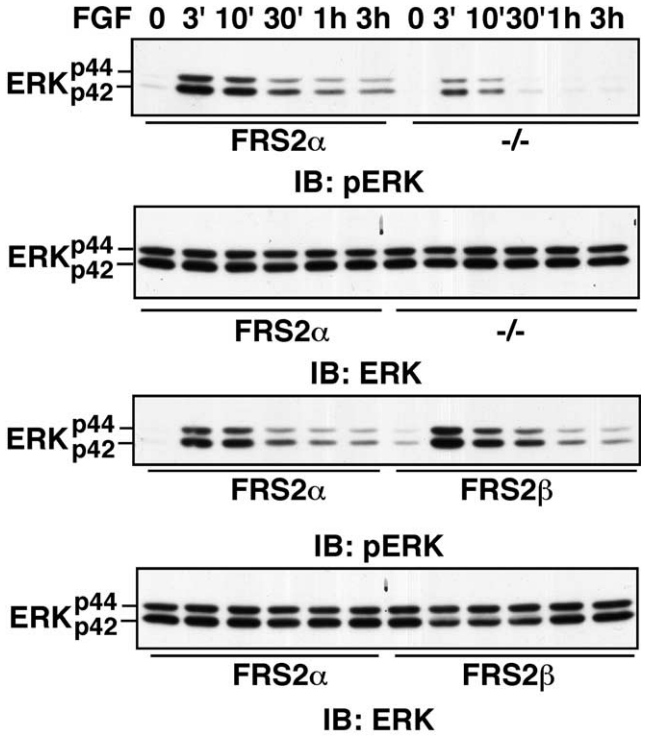

Fig. 4. Rescue of FGF-induced MAP kinase stimulation in FRS2α-deficient cells by ectopic expression of FRS2β. *Frs2α*$^{-/-}$ MEFs expressing FRS2α or FRS2β or vector-transfected *Frs2α*$^{-/-}$ were starved overnight and stimulated with FGF1. Lysates from unstimulated or stimulated cells were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotted with anti-phospho-Erk or anti-Erk antibodies.

ported previously, FGF-induced stimulation of MAP kinase in vector-transfected *Frs2α*$^{-/-}$ cells was weak and transient (Fig. 4, top panel), whereas FGF stimulation of *Frs2α*$^{-/-}$ cells expressing FRS2α resulted in rapid and sustained activation of MAP kinase (Fig. 4, top and third panels) [6]. Similarly, a strong and sustained MAP kinase response was observed in FRS2β-expressing *Frs2α*$^{-/-}$ cells (Fig. 4, third panel). Moreover, the MAP kinase response was somewhat more robust in cells expressing FRS2β in comparison with that of cells expressing FRS2α. Similarly, FGF-induced MAP kinase activation mediated by FRS2β was more robust than that mediated by FRS2α in HEK 293 cells (Fig. 3B, bottom panel). We also expressed two previously described chimeric proteins designated FRS2α/β and FRS2β/α in HEK 293 cells [9]. The former has the PTB domain of FRS2α and the C-terminal region of FRS2β, and the latter has the PTB domain of FRS2β and the C-terminal region of FRS2α. We found that MAP kinase activation was stronger in cells expressing FRS2α/β or FRS2β than in cells expressing FRS2β/α or FRS2α (Fig. 3B, bottom panel). These findings indicate that FRS2β can compensate for the loss of FRS2α in mediating the MAP kinase response to FGF stimulation and that FRS2β may yield a stronger MAP kinase response than does FRS2α in response to FGF stimulation.

FRS2β, unlike FRS2α, is not phosphorylated on threonine residues in response to FGF stimulation because it lacks canonical MAP kinase phosphorylation sites (Fig. 3B) [9]. The sustained activation of MAP kinase stimulation seen in *Frs2α*$^{-/-}$ MEFs expressing FRS2β is particularly striking as the expression of FRS2β was much lower than the expression of FRS2α in those cells (data not shown). It is possible that the loss of MAP kinase-induced negative feedback on FRS2β enables FRS2β to function significantly at lower levels of expression and to transmit a stronger signal for activation of MAP kinase than does FRS2α in response to growth factor stimulation.

In the present study, we found that FRS2α and FRS2β have overlapping roles in FGF-induced activation of MAP kinase, although FRS2β may activate MAP kinase more strongly than does FRS2α. In contrast, expression of the transcripts for these proteins is regulated developmentally in a distinct manner.

Our results suggest that FRS2α is involved in the development of multiple tissues, whereas FRS2β activity appears to be limited to development of a few tissues, including those of the nervous system. The localization of *Frs2α* in the VZ of the developing nervous system is particularly interesting, because this layer contains neural stem cells that can self-renew in response to FGF [14] (Figs. 1G, 2A, C). FRS2β may play a more significant role in the development of thalamus and hypothalamus than does FRS2α (Fig. 2I, J).

In conclusion, we propose that the distinct spatial and temporal patterns of expression of FRS2α and FRS2β dictate their physiological role(s) in vivo and that FRS2α and FRS2β mediate overlapping cellular responses following growth factor stimulation.

Acknowledgements: We thank Dr. M. Ito for critical reading of the manuscript. N.G. is a recipient of Research Fellowship from Uehara Memorial Foundation.

References

[1] Schlessinger, J. (2000) Cell 103, 211–225.
[2] Kouhara, H., Hadari, Y.R., Spivak-Kroizman, T., Schilling, J., Bar-Sagi, D., Lax, I. and Schlessinger, J. (1997) Cell 89, 693–702.
[3] Ong, S.H., Guy, G.R., Hadari, Y.R., Laks, S., Gotoh, N., Schlessinger, J. and Lax, I. (2000) Mol. Cell. Biol. 20, 979–989.
[4] Ong, S.H., Hadari, Y.R., Gotoh, N., Guy, G.R., Schlessinger, J. and Lax, I. (2001) Proc. Natl. Acad. Sci. USA 98, 6074–6079.
[5] Xu, H., Lee, K.W. and Goldfarb, M. (1998) J. Biol. Chem. 273, 17987–17990.
[6] Hadari, Y.R., Gotoh, N., Kouhara, H., Lax, I. and Schlessinger, J. (2001) Proc. Natl. Acad. Sci. USA 98, 8578–8583.
[7] Basilico, C. and Moscatelli, D. (1992) Adv. Cancer Res. 59, 115–165.
[8] Bibel, M. and Barde, Y.A. (2000) Genes Dev. 14, 2919–2937.
[9] Lax, I., Wong, A., Lamothe, B., Lee, A., Frost, A., Hawes, J. and Schlessinger, J. (2002) Mol. Cell 10, 709–719.
[10] Nakashima, M., Toyono, T., Akamine, A. and Joyner, A. (1999) Mech. Dev. 80, 185–189.
[11] Wassarman, K.M., Lewandoski, M., Campbell, K., Joyner, A.L., Rubenstein, J.L., Martinez, S. and Martin, G.R. (1997) Development 124, 2923–2934.
[12] Hadari, Y.R., Gotoh, N., Kouhara, H., Lax, I. and Schlessinger, J. (2001) Proc. Natl. Acad. Sci. USA 98, 8578–8583.
[13] Kaufman, M.H. (1992) The Atlas of Mouse Development, Academic Press, San Diego, CA.
[14] Davis, A.A. and Temple, S. (1994) Nature 372, 263–266.