in vivo *18*: 643-648 (2004)

Expression of JNK Interacting Protein JIP-1 is Down-regulated in Liver from Mouse Embryos with a Disrupted Insulin-like Growth Factor II Gene

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Abstract. *JNK interacting Protein 1 (JIP-1) is a pivotal scaffolding protein in the JNK signalling pathway. Its expression pattern in murine tissue indicates that JIP-1 has a role in the regulation of different cellular events. By examining the JIP-1 expression in transgenic mice that were heterozygous for a functional insulin-like growth factor II (igf-2) gene, it was possible to show that an abrogated igf-2 expression was followed by a decreased transcription of the JIP-1 gene. This pattern was consistent through different litters, which suggests that the up- or down-regulation of JIP-1 may well be part of the intracellular mediation of IGF II induced messages.*

Mitogen Activated Protein Kinases (MAPKs) play a central role in the intracellular mediation of signals for a plethora of cellular events such as proliferation, growth, differentiation, locomotion, survival and oncogenic transformation. Several extracellular effectors like growth factors, hormones and cytokines are capable of activating different intracellular MAPK cascades. In addition, stress factors such as irradiation, heat shock and disturbed osmotic balance can elicit a cellular response *via* the MAPK pathway (1,2).

In this pathway interest has been focussed on a set of key proteins – the c-Jun amino terminal kinases (JNK). These proteins have been shown to play a pivotal role in the control of early embryonic development in *Drosophila* (3). In mammals, JNK proteins play a more diverse role and are nowadays believed to be involved in oncogenic transformation. Human lymphocytes transformed *in vitro* by HTLV-1 show a constitutive activation of the JNK cascade (4). Moreover, p21, a cell cycle inhibitory protein, physically interacts with JNK and thereby inhibits its action (5). JNK has also been found to affect cell survival (6). When a cell is exposed to irradiation or alkylating agents, the JNK pathway is activated and eventually undergoes apoptosis. This mechanism seems to be prevented by the protooncogene Bcl-2. Interestingly, overexpression of JNK abrogates the immortalisation induced by Bcl-2 (7).

JIP-1 (JNK Interacting Protein 1) was discovered on the basis of its ability to interfere with JNK (8). Initially JIP-1 was described as a cytoplasmic protein that binds to JNK to prevent its localisation to the nucleus (8). Subsequently, it was found that JIP-1 binds several members of the JNK cascade, indicating that it functions as a scaffolding protein for the JNK signalling pathway (9) (Figure 1). The mechanism of scaffolding and anchoring adds another level of specificity to the intracellular signalling system, since it co-ordinates the binding of specific proteins and consequently elicits a specific response (10). Mutations in the JIP-1 gene result in an inadequate regulation of the JNK transduction and consequently give rise to various pathological conditions, including certain forms of cancer (8).

Since JIP-1 appears to play an important role in intracellular signal transduction (11,12), we aimed to study whether the expression of the JIP-1 gene is in any way related to somatic growth. It has been known for some time that the insulin-like growth factor II (IGF II) is the most ubiquitous growth factor in the mammalian embryo. Inactivation of the IGF II gene (igf-2), or ,more particularly, when the paternal allele was inactivated by homologous recombination in transgenic mice, resulted in 40% overall growth inhibition (13). Since the igf-2 gene is parentally imprinted (14), the maternal igf-2 gene is constitutively silent and does not require experimental inactivation for this purpose. We used mice that were heterozygous for the igf-2 mutation as a model for our studies of JIP-1 expression in different genotypes. It was found that, in each of the mutated embryos, the transcriptional rate was substantially lower than in their wild-type counterparts.

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Key Words: JIP-1, IGF II, mouse embryos.

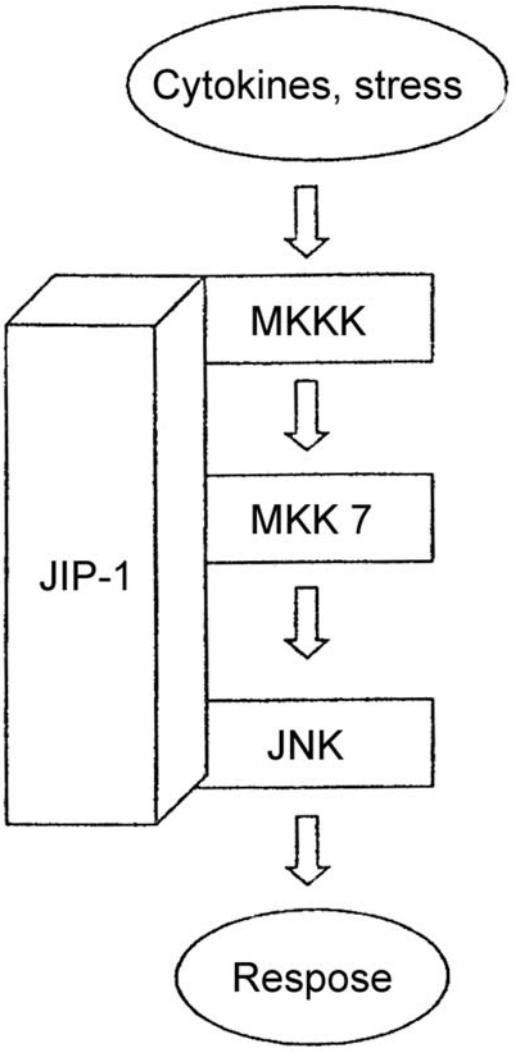

Figure 1. *A model over JIP-1 functioning as a scaffold that binds to members of the MAPK cascade for selective regulation of JNK activation.*

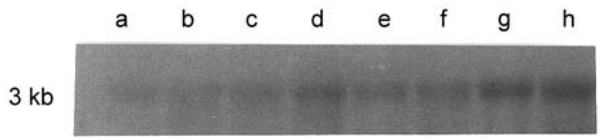

Figure 2. *Expression of the JIP-1 gene in normal fetal mouse tissue. A pre-prepared embryoblot was hybridised with an JIP-1 cDNA-fragment. The lanes represent; a) brain E11.5 b)gonads E14.5 c) heart E12.5 d) intestine E14.5, e) kidney E13.5 f) liver E 11.5 g) lung E12.5 h) spleen E15.5.*

Materials and Methods

Primary material and genotyping. The genotype of the animals were the igf-2 wild-type ($igf2^{m+/p+}$) and heterozygous mutants ($igf2^{m+/p-}$). The first mice were obtained from Dr A. Efstradiatis (13, 14) and subsequently mated and bred in the Department of Zoology, University of Oxford, U.K. Twelve litters born in 2001 were genotyped and three of them (Litters 1,2 and 6) sacrificed on day E14.5 and the livers removed and used for this study.

Briefly, the gene disruption had been achieved using the neomycin phosphotransferase resistance gene (Neo). Polymerase chain reaction primers were designed to detect any of the three possible genotypes. In the original gene disruption mutants, a 1.6 kB fragment of the wild-type igf-2 gene was acquired after digestion with restriction endonuclease BstE II, which cleaves at a GGTnACC sequence (position 21026) at the end of exon E1.2, and restriction nuclease BamH I, which cleaves at a GGATCC sequence (position 22603) immediately downstream of the ATG codon in exon E2. This resulted in a fragment with two palindromic ends containing single-stranded DNA, that was further treated with mung bean nuclease resulting in a 1.6 kb fragment with blunt ends. Similarly, the Neo gene was cleaved from the vector pMC1 using restriction enzymes BamHI and XhoI, which cleave at CGATCC and CATCGAG sequences, respectively, also leaving palindromic ends that were cut back to blunt ends. Finally, an 8.3 kb fragment of the wild-type genome was produced by digestion with a SmaI restriction nuclease, which cleaves at CCCGGG sequences (position 22866) leaving blunt ends. The 3' end of this fragment was joined to the thymidine kinase (tk) gene.

The 1.15 kb Neo fragment, 1.6 kb igf-2 fragment and the 8.3 tk-containing fragment were joined by blunt end ligation. The complete 11.05 fragment was then introduced into embryonic stem cells *via* homologous recombination resulting in the replacement of the 0.25 kb wild-type sequence, including exon 2 and 0.1 kb of the downstream intron, with the larger 1.15 kb Neo fragment. Positive selection was performed using the drug G418 that destroyed all non-Neo incorporating cells, thus enriching the cell lines containing the Neo fragment. Negative selection by utilising Gancyclovir in the presence of thymidine kinase is lethal to cells. This would exclude cells which had random integration of the complete vector and those with episomal fragments. Only cells with the correct fragment incorporation, that had lost the tk-gene during homologous recombination, survived.

The only 5'- 3'primer is an Exon 1.2 primer which is a 20 bp oligonucleotide (position 22509), (5'- GCC TGG GTG GGG GTG AGC CA – 3'). In the wild-type the Exon 1.2 primer will read with the 3'- 5'- primer (position 22646), (5'- CAA AGA GAT GAG AAG CAC CA – 3'). The products primers including the total 40 primer bases are calculated to be 137 bp. If the wild-type igf-2 sequence had been replaced by the Neo-fragment, the 5'-3' primer E1.2 will read with the 3'-5' Neo primer , also a twenty base primer, (5'-TCG-GCT-GAC-AGC-CGG-AAC-AC-3'). This primer begins at position 415 downstream of the beginning of the neo fragment, *i.e.* at the BamHI site. Therefore, the size of the transgenic PCR product is calculated to be ((22603(BamHI site) - 22509 (E1.2 site)) + 415= 509 bp.

Polymerase chain reaction. Igf-2 gene disruption PCR reactions were run on a Cyclogene Techne Thermo Cycler. The reaction was initiated with a 2 min hot start at 94˚C, after which Taq Polymerase (Advanced Biotechnologies,Stockholm, Sweden Cat No 0315) was added. The conditions were; 94˚C 1 min denaturation, 58.5˚C 1 min annealing and 72˚C for 45 sec elongation for 35 cycles. Magnesium chloride was used at 1 mM, dNTPs at 0.2 mM each, primers at 25 pmols each and reaction buffer at 1 x.1.75 µl of DNA from fetal extracts was added per 100 µl reactions.

DNA extraction from fetal tissues. Fetal livers were removed and stored at -20˚C. For DNA extraction, the liver samples were placed in a 1.5 ml microfuge tube where 300µl of Tail Buffer (50 mM Tris pH 8, 100 mM

Table I. Summary of the genotypimg of twelve different litters born by a homozygous (Igf-2$^{+/+}$) female bred onto a heterozygous (Igf-2$^{+/-}$) male mouse. The litters are consecutively numbered and litters 1,2 and 6 were used for further analysis.

Litter no	Total number of offspring	Igf-2$^{+/+}$	Igf-2$^{+/-}$
1	6	1	5
2	3	1	2
3.	5	3	2
4	8	5	3
5.	7	3	4
6.	4	2	2
7.	7	3	4
8.	8	2	6
9.	10	7	3
10.	7	4	3
11.	7	2	5
12.	6	3	3
Total	78	36	42

EDTA, 100 mM NaCl and 1% SDS) and Proteinase K at 100µg/ml was added. This was then incubated overnight at 55˚C

On day two, 2 µl of 0.2 mg/ml RNase A was added and, after 1 h incubation at 37˚C, 300 µl of 5M LiCl was added and gently mixed for 1 min. Six hundred µl of Chloroform:Isoamylalcohol (24:1) was next added and the tubes were placed on a rotary mixer for 1 h at room temperature. The tubes were then spun at 10000 g for 10 min to produce two layers; the lower layer of chloroform:isoamylalcohol and cellular debris and an upper aqueous layer containing the DNA extracts. Four hundred µl of this upper layer was then carefully removed so as not to include any of the lower layer and placed into another 1.5 ml microfuge tube. To this, 400 µl of isopropanol was added to precipitate the DNA from solution. The tube was spun again at 15700 g for 10 min to pellet the DNA, after which the Isopropanol was removed and replaced with 300 µl of 70% alcohol and left for 15 min at room temperature for pellet cleansing. Finally, the sample was briefly spun, the alcohol was removed and the tubes placed in a 37˚C room for 1 h to dry the sample. This was followed by re-suspension with 100 µl TE buffer (1M Tris and 0.5M EDTA in dH_2O) and storage at 4˚C. After 24 h, these samples were ready for PCR analysis.

RNA extraction and Northern blotting. Total embryonic liver RNA from the experimental mice was extracted by a standard Trizol/Chloroform extraction procedure. In each case the quality was checked by running the samples on an ethidium bromide-containing minigel. Moreover, in each case quantitation was carried out by spectrophotometry. From these total RNA samples, polyadenylated RNA was purified using an Oligo dT cellulose-based purification technique described (15). One µg of polyadenylated RNA from the liver from each embryo in litters 1,2 and 6 (Table I) was run on a denaturing agarose/formaldehyde gel (15). The electrophoresed RNA was then transferred by blotting onto Hybond N+ filter (Amersham Pharmacia Biotech, Sweden), crosslinked by UV light and stored in a sealed plastic bag until further use. To examine the basic JIP-1 expression in normal fetal tissues, we used a pre-prepared embryoblot which contained fetal and normal murine tissues. The EmbryoBlot was a kind gift from Quantum-Appligene (Illkirch, France).

Table II. *Summary of findings from three different litters born by homozygous (Igf-2$^{+/+}$) females bred onto a heterozygous (Igf-2$^{+/-}$) male mouse. The table summarises, for each embryo, the size determined as crown-rump (C-R) length in cm, the IGF genotype as determined by PCR and the relative expression of the Igf-2 and JIP-1 genes (arbitrary values). Finally, the relationship between igf-2 and JIP-1-expression was calculated as a quota based on densitometric measurements of X-ray films.*

Litter 1

Embryo	IGF II genotype	C-R (cm)	Igf-2 expr.	JIP-1 expr	Igf-2/ JIP-1
1.	+/-	1.0	(+)	(+)	1.3
2.	+/-	1.1	(+)	(+)	1.1
3.	+/-	0.8	-	+	-
4.	+/+	1.6	+++	+++	1.0
5.	+/-	1.1	++	++	0.9
6.	+/-	1.1	(+)	(+)	0.9

Litter 2

Embryo	IGF II genotype	C-R (cm)	Igf-2 expr.	JIP-1 expr	Igf-2/ JIP-1
1.	+/+	1.3	+++	+++	1.0
2.	+/-	1.0	-	+	-
3.	+/-	1.0	+	++	0.8

Litter 6

Embryo	IGF II genotype	C-R (cm)	Igf-2 expr.	JIP-1 expr	Igf-2/ JIP-1
1.	+/+	1.6	+++	+++	1.0
2.	+/-	1.1	(+)	+	1.1
3.	+/+	1.7	+++	+++	1.0
4.	+/-	1.0	(+)	(+)	0.9

cDNA probes , radioactive labelling and hybridisation. For the analysis of gene expression two probes were used; a 500 bp mouse Igf-2 coding sequence cDNA (from Dr A Shokrai, Uppsala, Sweden) and a 2832 bp murine JIP-1 cDNA fragment (13). The cDNA:s were labelled with 32P-dCPT by using a Megaprime DNA labelling system (Amersham Life Science) The filters were hybridised in a prefabricated hybridisation buffer supplied by Amersham Pharmacia Biotech (Sweden) as described by the manufacturer's instructions. After hybridisation, the filters were washed to a stringency level of 0.1 x SSC, 55˚C, air dried and subjected to autoradiography. To obtain a comparable value of the relative JIP-1 and igf-2 expression, each film was subjected to

densitometry. The integrated igf-2 and JIP-1 values were divided by each other and the quota taken as a relative expression measure.

Statistics. The statistical difference between means was calculated by Student's *t*-test. The level of significance was set at $p=0.05$

Results

Figure 2 shows the expression of JIP-1 in different fetal mouse tissues. The experiment shows conclusively that the JIP-1 gene is expressed in fetal brain, gonads, heart, intestine, kidney, liver, lung and spleen, albeit at different levels. To examine the JIP-1 expression in genetically-engineered mice, fifteen female wild-type mice were mated with male heterozygotes (igf-$2^{+m/-f}$) Of these twelve developed into pregnancy and produced embryos of wild-type as well as igf-$2^{+m/-f}$ genotype. Three pregnant mice (1, 2 and 6 (Table I)) were sacrificed. All embryos were measured from crown to rump to get an approximate estimation of the size of each litter member. Also, a small sample was taken for analysis of the presence of the IGF II transgene. It was found that, in each of the litters, the wild-type offspring were significantly ($p<0.05$) larger than the igf-$2^{+/-}$ embryos. The data on the mice from litters 1, 2 and 6 are summarised in Table II. All offspring in the remaining litters were genotyped for comparison (Table I). It was found that, out of a total number of 78 embryos, 36 were wild- type and 42 were igf-$2^{+/-}$ heterozygotes.

Litter 1 consisted of one wild-type embryo and five heterozygotes; litter 2 of one wild-type and two heterozygotes. Litter 6 consisted of four embryos, of which two were wild-type and two heterozygotes. Next, each embryo was dissected and the livers used for the extraction of polyadenylated RNA. Northern blotting was performed with igf-2 and JIP-1 cDNA as probes. The expression of the two genes in relationship to a control housekeeping gene was quantified by densitometry and expressed as abitrary values (Table II). It was found that the transcription of the igf-2 gene was not completely switched off in all igf-$2^{+/-}$ embryos. As expected the expression was high in the wild-type embryo (litter 1, embryo 4, litter 2, embryo 1 and litter 3, embryos 1 and 3) but varied considerably between the knockout embryos. In litter 1, embryos number 1, 2 and 6 had igf-2 expression levels that were barely traceable. Embryo no 3 was negative for igf-2 transcripts, whereas embryo 5 had a visible but subnormal transcription of the igf-2 gene. In litter 2, embryo 2 did not display any expression of igf-2 and very low levels of JIP-1. Embryo 3 had a weak igf-2 expression and a moderate expression of JIP-1. Litter 3 was the only litter with an expected Mendelian outcome consisting of two wild-type offspring and two heterozygotes. Both heterozygotes had a very weak expression of igf-2, but the JIP-1 expression was slightly higher in embryo 4 as compared to embryo 2.

To make a valid comparison of the expression pattern of the two genes in wild- type and heterozygote embryos, each filter was hybridised with an igf-2 cDNA, and then stripped of bound probe and rehybridised with JIP-1 cDNA. The intensity of the relevant bands were examined after both hybridisations by densitometry and the integrated absorption values divided by each other. All figures were normalised by multiplication with a constant so that the wild-type relationship in each litter was given the relative value 1 (16). It was found that in none of the embryos – irrespective of genotype – did the quota between igf-2 and JIP-1 expression differ significantly from each other ($p<0.05$). This suggests that the magnitude of expression of JIP-1 in genetically-engineered mice is closely related to the magnitude of igf-2 expression. The relative values were all in the range 0.8-1.3, which indicates a closely controlled relationship between the transcriptional patterns of the two genes.

Discussion

Recent evidence suggests that most intracellular signals are not transmitted in a linear stepwise fashion from one intermediary to another. Instead, individual proteins tend to associate in networks where their activity is regulated by many independent events. Moreover, even membrane receptors seem to collaborate with each other, for instance by dimerisation upon activation. Receptors activate specific signalling proteins: for instance Src, other tyrosine kinases, phosphatidyl inositol kinases, small GTP:ases as *e.g.* Rac, Rho and Ras) and MAPKs. Each of these is activated by multiple, structurally different receptor families in order to elicit distinct and specific biological responses (reviewed in 17).

It is conceivable that any attempt to understand the mechanisms that control the receptor and cell specificity of these kinases will help elucidate the principles of intracellular transduction. The MAPK pathway anchors individual kinases to a protein scaffold, which controls the timing and sequence of events and adds to the specificity of the pathway. The JIP-1 protein, which is one of the key scaffold proteins, was originally isolated by means of its ability to bind JNK proteins (8). Quite unexpectedly, the JIP-expression differed between different tissues in the adult mouse (8) thereby indicating some important growth regulatory role of this protein. This was partly followed-up by the demonstration of JIP-1 transcripts in 2-cell embryos and blastocysts (18). We show, in this paper, that the tissue-related differences observed in adult mice are less augmented in the mouse embryo. Even though there are quantitative differences in JIP-1 expression, each examined organ showed at least a minimum transcriptional activity.

It, therefore, became of interest to examine whether these observations might reflect differences in growth signalling. Since growth regulatory messages are often mediated by polypeptide growth factors, we attempted to examine if the JIP-1 gene expression is in any way related to the activation of a key growth factor. For this type of analysis IGF II was

chosen, because of its high rate of transcription in the normal embryo where the JIP-1 gene is also expressed. We took advantage of a set of mice where the paternal igf-2 allele had been replaced by a non-functional igf-2 / Neo gene trap. By breeding heterozygous igf-$2^{+/-}$ males onto wild-type females, a mixed litter containing igf-$2^{+/+}$ and igf-$2^{+/-}$ offspring could be produced. Since the IGF II gene is parentally imprinted (14), igf-$2^{+/-}$ mice will in principle not express this gene. The key finding of this study is that igf-$2^{+/-}$ mice also have a lower expression of the JIP-1 gene, indicating that a reduced transcription of igf-2 results in a concomitant decrease in JIP-1 expression. This co-variation indicates the possibility that JIP-1 is part of the IGF II signal transduction pathway. Somewhat surprisingly, we found that, in some of the igf-$2^{+/-}$ embryos, there was some low-level igf-2 expression indicating that the knockout was not complete. One distinct possibility is that some, yet undescribed, form of up-regulation of the normally imprinted (silent) maternal allele exists. Alternatively, this low level igf-2 expression might simply reflect the activity in tissues (as *e.g.* the leptomeninges) where the imprinting of the igf-2 gene is relaxed and transcription progresses in an biallelic fashion (19-21). Nevertheless, it was found consistently in this study that even a partial reduction in igf-2 expression was always accompanied by a lowering in JIP-1 transcription. It thus remains to be shown how other members of the MAPK cascade are affected by interference with the embryonic igf-2 expression. This work is currently in progress.

Acknowledgements

This study was supported by Cancerfonden, Barncancerfonden, the Swedish Medical Research Council and the Cancer Research Campaign of Great Britain. We acknowledge an UICC travelling fellowship. The authors are grateful to Dr RJ Davis for the gift of the JIP-1 cDNA probe and to Professor Christopher Graham FRS and Dr Andrew Ward for stimulating discussions.

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Received March 10, 2004
Revised July 28, 2004
Accepted August 9, 2004