Journal of Cell Science 112, 3967-3974 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
JCS0496

Pattern of expression of the Jun family of transcription factors during the early development of the inner ear: implications in apoptosis

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Accepted 1 September; published on WWW 3 November 1999

SUMMARY

Jun transcription factors have been implicated in the regulation of cell proliferation, differentiation and apoptosis. We have investigated the relationship between Jun expression and cell death in the developing chicken inner ear. c-jun and junD transcripts were expressed in the epithelium of the otic placode and otic vesicle. c-jun expression was restricted to the dorsal area of the otic pit (stages 14-17), dorsal otic vesicle and cochleo-vestibular ganglion (stages 18-20). junD expression was transient and occurred in the dorsal and upper medial aspects of the otic pit and otic cup, but it was down-regulated in the otic vesicle. A parallel TUNEL analysis revealed that expression of c-jun co-located within areas of intense apoptosis. Furthermore, phosphorylation of c-Jun at serine-63 by Jun amino-terminal-kinases was detected in the dorsal otic pit, otic vesicle and cochleo-vestibular ganglion. c-Jun protein exhibited DNA binding activity, as assessed by gel mobility shift assays. The association between c-Jun and apoptosis was further demonstrated by studying nerve growth factor-induced apoptosis in cultured otic vesicles. Nerve growth factor-induced cell death and c-Jun phosphorylation that were suppressed by insulin-like growth factor-I and by viral-mediated overexpression of Raf, which had survival effects. In conclusion, the precise regulation of the expression and activity of Jun proteins in the otic primordium suggests that it may operate as a fundamental mechanism during organogenesis.

Key words: IGF-I, Jun family, NGF, JNK, Otic vesicle, RCAS vector, Sensory organ

INTRODUCTION

Transcription factors are central to achieving specificity of the cellular responses to extracellular signals. Jun proteins are major components of the AP-1 transcription factor and show tissue-specific and differential expression during embryonic development (Wilkinson et al., 1989; Angel and Karin, 1991). c-Jun, JunB and JunD differ in their DNA-binding properties, displaying distinct and sometimes opposite roles (Ryseck and Bravo, 1991; Castellazzi et al., 1991). c-jun knock out mice die in utero and cells lacking c-jun show retarded growth in culture, suggesting that c-jun is critical for the control of cell proliferation (Johnson et al., 1993). On the other hand, Jun signalling has been implicated in the regulation of cell differentiation (Treier et al., 1995) and apoptosis (Marti et al., 1994; Eilers et al., 1998). Jun proteins are activated by phosphorylation through two distinct pathways: first, survival factors induce the activation of certain mitogen-activated kinases (MAPKs) which induce c-jun expression and stimulation of cell proliferation. Secondly, the activation of another set of MAPKs, the Jun amino-terminal-kinases (JNKs), leads to apoptosis (Karin et al., 1997). JunD is phosphorylated less efficiently than c-Jun while JunB is not phosphorylated by JNKs. This indicates that not only the expression but also the specific phosphorylation of Jun may determine the functions played by the different Jun proteins.

The precise role of Jun in the regulation of cell death, survival and differentiation during embryonic development is still poorly understood. The molecular correlates of early developmental decisions in ear ontogenesis are beginning to be identified, providing an attractive model system to study the specificity of AP-1 signalling. Insulin-like growth factor-I (IGF-I) and nerve growth factor (NGF) regulate cell number and cell differentiation in the developing inner ear (Torres and Giráldez, 1998; Frago et al., 1998). IGF-I activates the Raf/MAPKs pathway, and increases Fos and Jun levels (Sanz et al., 1999). Antisense c-fos and c-jun oligonucleotides partially block the proliferative effect of IGF-I (León et al., 1995a, 1998). These results indicate that Fos/Jun heterodimers govern proliferative growth of the otic vesicle. However, in cultured otic vesicles the expression of c-jun transcripts coincides with areas of NGF-induced cell death (Frago et al., 1998), suggesting that Jun proteins may play a dual role during early inner ear development.

Here we show the presence of c-jun and junD transcripts in the otic pit, otic vesicle and cochleo-vestibular ganglion (CVG), along with the phosphorylation of c-Jun at serine-63 residue within the same areas during the early stages of inner

ear development. The functionality of c-Jun protein was established by determining its ability to interact with an specific AP-1 recognition site. Mapping of apoptotic regions revealed that c-Jun expression and phosphorylation domains overlapped with areas of high apoptotic activity. Furthermore, NGF increases c-Jun phosphorylation in parallel with the induction of apoptosis in cultures of explanted otic vesicles, and this increase is inhibited by IGF-I or viral-mediated overexpression of Raf. These results suggest that Jun transcription factors may specify proliferative and apoptotic areas within the otic vesicle and link extracellular signals to long-term cellular responses during organogenesis.

MATERIALS AND METHODS

Materials

Recombinant NGF was from UBI (Lake Placid, NY). Human IGF-I was purchased from Boehringer Mannheim (Mannheim, Germany). Anti-phosphorylated-c-Jun (KM-1) is a mouse monoclonal IgG1 antibody raised against a peptide corresponding to amino acids 56-69 of human c-Jun, it reacts with c-Jun p39 phosphorylated at serine-63 of different species. It shows no cross-reactivity with JunB or JunD phosphorylated on the analogous serine residues or with c-Jun non-phosphorylated at serine-63 (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Anti-c-Jun (sc-45X) used for gel supershift assays was from Santa Cruz. The Cell Death Detection and In situ Cell Death Detection-POD kits were from Boehringer Mannheim.

Chicken embryos were obtained from fertilised eggs (Granja Rodríguez Serrano, Salamanca, Spain) that were incubated at 38°C in a humidified atmosphere. The embryos were staged according to Hamburger and Hamilton criteria (1951). Animal care and handling complied with the guidelines of the European Community directive (86/69/CEE) and the Spanish legislation (R.D. 223/1988) and was approved by the Committe of Biosafety and Animal Care of the IIB.

Otic vesicle isolation, cell culture and production of viral stocks

Otic vesicles were dissected from embryos corresponding to stage 18 using a dissection stereomicroscope (Nikon SMZ-2T), transferred into four-well culture plates (NUNC Roskilde, Denmark) and incubated at 37°C in a water-saturated atmosphere containing 5% CO_2 as described (Miner et al., 1988; León et al., 1995b, 1998). The standard culture medium consisted of serum-free M199 medium with Earle's salts (Biowhittaker, Walkersville, MD) supplemented with 2 mM glutamine (Biowhittaker), and antibiotics (penicillin, 50 i.u./ml and streptomycin, 50 mg/ml) (Biochrom, Berlin, Germany). FBS was purchased from Biowhittaker.

Line 0 CEF were prepared as described by Hunter (1979) from embryonic day 10 chicken and cultured in growth medium (DMEM, 10% FBS, 2% chicken serum, penicillin/streptomycin and glutamine as above).

RCAS is a replication-competent retroviral vector derived of Rous sarcoma virus (Hughes et al., 1987). It contains a *Cla*I site for cloning of exogenous genes. RCAS envelope subgroup A vector was the generous gift of Dr S. Hughes (National Cancer Institute-Frederick, MD). c-raf cDNA (gift from Dr U. Rapp, University of Würzburg, Germany) (Heidecker et al., 1990) was inserted into RCAS at the *Cla*I site as described by Sanz et al. (1999) and RCAS clones containing the c-raf inserts in the sense orientation (RCAS/RAF) were identified and isolated. CEF were transfected with 10 mg of the plasmid containing the provirus by calcium phosphate co-precipitation (Petropoulos and Hughes, 1991). Transfected cells were then passaged and expanded for 10 days in CEF culture medium; at the end of that period all cells were infected, as estimated by immunohistochemistry with anti-p27 antibody that was a generous gift of Dr Volker M. Vogt (Cornell University, NY). Viral supernatants were collected in culture medium on the following two days and concentrated 100-fold by centrifugation (3 hours, 28,000 rpm). Then, the titer of the viral stock was determined as described by Fekete and Cepko (1993). Positive colonies were identified and counted to obtain the titer that is defined as colony-forming units per ml (cfu/ml). Titers were typically in the range $2\text{-}3\times10^8$ cfu/ml. Two viral preparations were used for these studies with similar results.

Infected-CEF cells were plated in cell culture inserts to a density of 8×10^4 cells/insert and co-cultured with otic vesicle explants at 37°C and 5% CO_2 for 24 hours as described by Sanz et al. (1999). The degree of infection was estimated by western blotting or immunohystochemistry with anti-p27 antibody. When indicated, explants were cultured for a further period of 16 hours in the presence or absence of 1 nM IGF-I or 4 nM (100 ng/ml) NGF. Otic vesicles were homogenised individually and the cell extracts subjected to enzyme-linked immuno-assay (ELISA) determination (Frago et al., 1998). In parallel, five explants per condition were fixed in 4% (w/v) paraformaldehyde and the levels of jun phosphorylation determined by immunohistochemistry.

Whole mount in situ hybridisation

In situ hybridisation was performed as described by Goulding et al. (1994) with the modifications reported by León et al. (1998) using digoxigenin-labelled RNA chicken probes for c-jun (Bos et al., 1990) and junD (Hartl et al., 1991) kindly provided by Drs M. Zenke (Max Delbrück Center, Berlin, Germany) and M. Hartl (University of Innsbruck, Austria), respectively. A single-stranded c-jun RNA antisense probe was prepared by the transcription of the linearised plasmid pGEM7Z-CJ-3 with *Eco*RI using SP6 RNA polymerase (Promega, Southampton, UK). Control sense probe was prepared by linearising the plasmid with *Bam*HI and using T7 RNA polymerase (Promega). An *Eco*RI fragment of JunD was extracted from the plasmid JunD-bluescript and inserted in PAJ10 bluescript. The sense and antisense riboprobes were prepared by transcription of the linearised plasmid with *Bam*HI and *Hin*dIII and using T7 and T3 RNA polymerases, respectively. For hystological examination, after in situ hybridisation the embryos were post-fixed in 4% paraformaldehyde, embedded in gelatine-albumin and sectioned with a vibratome (Leica VT 1000 M, Heidelberg, Germany) at a thickness of 60 μm.

Immunohistochemistry

This procedure was performed on frozen sections obtained from at least ten embryos (stage 14 to 19). Whole embryos were fixed in 4% (w/v) paraformaldehyde and cryoprotected in sucrose. Embryos were coated with Tissue Tek (Sakura Finetek, Torrance, CA) and sectioned (20 μm, Leitz cryostat, Jena, Germany). Sections were placed on poly-L-lysine-coated slides and stored at −20°C until use. Sections were washed with 0.1 M PBS, pH 7.4, for 1 hour, treated for 30 minutes with 0.3% Triton X-100 in PBS and for a further period of 30 minutes with PBS, pH 7.4, solution containing 2% bovine serum albumin and 5% goat serum. Afterwards, sections were incubated overnight with an anti-phospho-c-Jun mouse monoclonal antibody (KM-1) (Santa Cruz Biotechnology) diluted 1:200 in PBS containing 2% bovine serum albumin and 5% goat serum. Sections were then processed with an anti-mouse antibody (EnVision, DAKO, Carpinteria, CA) and developed with the peroxidase reaction. Control sections were incubated with preimmune serum instead of the primary antibody and then processed as described above. Sections were dehydrated and mounted in the mounting medium Entellan (Merck, Darmstadt, Germany). Microphotographs were taken in a Zeiss microscope Axiophot. Computer-generated photomicrographs were produced by scanning the obtained slides with a Nikon CoolscanII using the programs Adobe Photoshop LE 2.5 and MacDraw Pro on Macintosh computers.

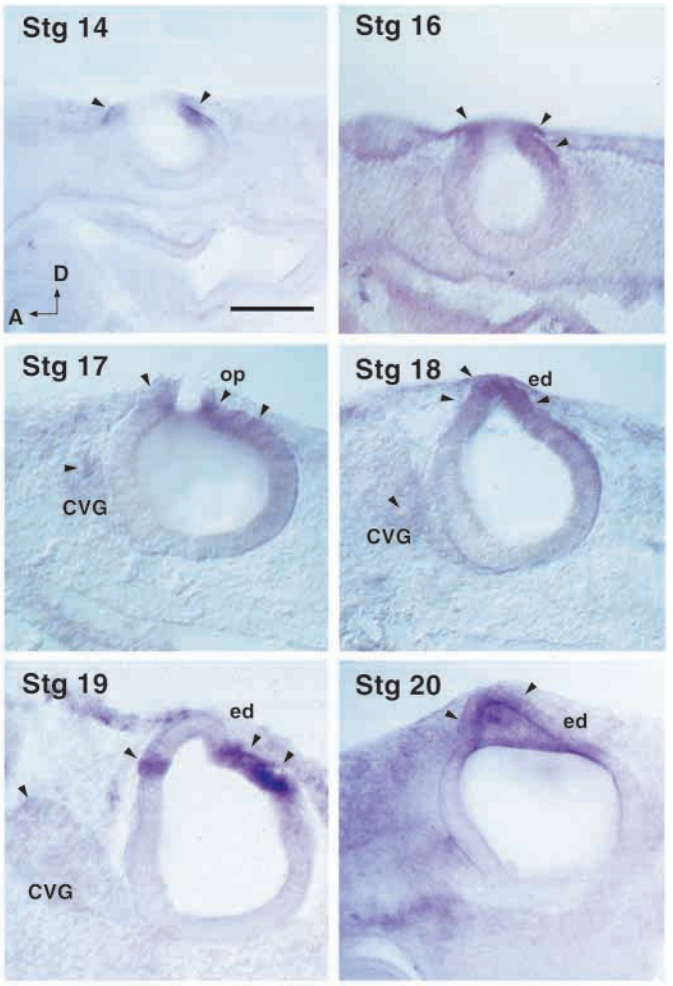

Fig. 1. Expression of c-jun during the early development of the inner ear. Microphotographs from vibratome sections obtained after whole mount "in situ" hybridisation. Chicken embryos between stages 14 to 20 were dissected and in situ hybridisation performed as described in Materials and Methods, using digoxigenin-labelled RNA chicken probes for c-jun. After hybridisation the embryos were post-fixed in 4% paraformaldehyde, embedded in gelatine-albumin and sectioned at 60 μm with a vibratome. Arrowheads point to the c-jun transcript expression. The pattern of c-jun expression shown is representative of 4 different experiments, with at least 5 embryos per stage. Bar, 150 μm. A, anterior; D, dorsal; op, otic pit; CVG, cochleovestibular ganglion; ed, endolymphatic duct.

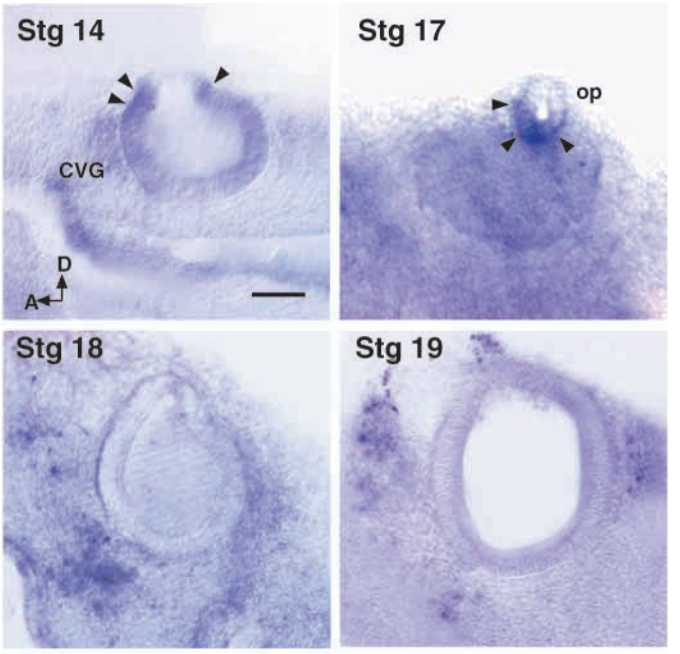

Fig. 2. Expression of junD in the otic placode and the otic cup. Chicken embryos were dissected at stages 14 to 19 and in situ hybridisation was performed as described, with a chicken junD probe. Photographs from 60 μm vibratome sections. Arrowheads point to the junD transcript expression. The experiment shown is representative of 3 performed with at least 5 embryos per stage. Bar, 100 μm. A, anterior; D, dorsal; op, otic pit; CVG, cochleovestibular ganglion.

Cell death determination by TUNEL staining and immunoassay

Distribution of apoptotic cells in the otic vesicle was determined by TUNEL staining as described by Sgonc et al. (1994). This procedure allows the detection of individual apoptotic cells in frozen tissue sections. Fragmented DNA was labelled by the terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labelling reaction according to the manufacturer's instructions (Boehringer Mannheim). Slides were then rinsed 3 times with PBS and incubated with the Converter-horseradish peroxidase (POD) solution (anti-fluorescein antibody conjugated with POD, Boehringer Mannheim). Apoptotic nuclei were visualised with DAB and analysed by light microscope with a Zeiss Axiophot Microscope.

Quantification of cell death was performed by using the Cell Death Detection ELISA from Boehringer Mannheim, basically as indicated by the manufacturer and described by Frago et al. (1998).

Preparation of nuclear extracts and electrophoretic mobility shift assay

Nuclear extracts were prepared from otic vesicles isolated from 100 embryos (stage 20). At this stage the CVG is attached to the otic vesicle, both structures were isolated and analysed together. The procedure followed was essentially as described elsewhere (Andrews and Faller, 1991), but 0.2% Triton X-100 was included in the hypotonic lysis buffer. Nuclear proteins were extracted with high salt buffer containing 20 mM Hepes, pH 7.9, 0.4 M KCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 1 mM dithiotreitol, 0.2 mM phenylmethylsulphonyl fluoride, 0.5 mg/ml leupeptin, 10 mg/ml soybean trypsin inhibitor, and 25% glycerol. Nuclear extracts were aliquoted and stored at −70°C until used. Protein concentration was determined with the Bradford protein assay kit (Bio-Rad).

The double-stranded oligonucleotide probes used in the electrophoretic mobility shift assay were jun1TRE (upper strand, 5′-AGCTGGGGTGACATCATGGG-3′) and jun2TRE (upper strand, 5′-AGCTAGCATTACCTCATCCC-3′) that are derived from the proximal and distal AP-1 binding sites of the human c-jun promoter, respectively (Angel et al., 1988; van Dam et al., 1993). The oligonucleotide 5′-GCTTTGGCATGCTGCCAATATG-3′, containing a consensus NF-1 binding site (Jones et al., 1987), was used as non-specific competitor. DNA binding assay was carried out in a 20-ml reaction containing 10 mg of otic vesicle nuclear proteins, 40 mM Hepes, pH 7.5, 80 mM KCl, 0.2 mM EDTA, 5 mM $MgCl_2$, 1.25% Ficoll, 1 mg poly(dI-dC).poly(dI-dC), 1 mg of sonicated herring sperm DNA, and, when indicated, a 50-fold molar excess of competitor oligonucleotide. The reaction mixtures were incubated on ice for 15 minutes, then 8×10^4 cpm of annealed oligonucleotides were

added and incubation continued for an additional period of 15 minutes. For gel supershift assays, nuclear proteins were incubated with either 0.5 mg or 2 mg of anti-c-Jun antibody for 30 minutes prior to the addition of the labelled probe. The DNA-protein complexes were resolved in a 5% polyacrylamide gel in 0.5× TBE (1× TBE: 89 mM Tris, pH 8.0, 89 mM boric acid, 2 mM EDTA).

RESULTS

Expression of Jun transcription factors

In stage 14, c-jun transcripts were expressed at the dorsal ridge delimiting the otic pit from the surrounding ectoderm (Fig. 1). In stages 14 to 17, as the otic pit invaginated, c-jun expression was maintained in the otic pore, with an intense dorsal and caudal expression domain. In stages 18, 19 and 20 (Fig. 1), c-jun expression was restricted to the dorsal epithelium, and it was also expressed in the CVG. Dorsal expression of c-jun seemed to extend through a narrow belt at the basis of the endolymphatic duct. Northern blot analysis performed on poly-A mRNA preparations of stage 20 otic vesicles revealed also a 2.7 kb transcript that was identified with a chicken c-jun probe (León et al., 1998). However, comparison of c-jun expression pattern with previously reported Jun induction and Jun immunoreactivity (see Discussion) suggested that, most probably, c-jun was not the singular member of the Jun family of factors expressed in the otic vesicle. Since junB has not been cloned from avian cells, the next step was to study the pattern of expression of junD.

junD was expressed in the otic placode and the otic cup. junD transcripts were present in the upper medial epithelium of the otic vesicle and in the CVG (Fig. 2). junD expression was transient and it was not detected from stage 18 onwards in the epithelium of the otic vesicle (Fig. 2, stage 18 and stage 19). Patches of junD expression associated with neural crest cell migration were also apparent. JunD expression still did not account for Jun immunoreactivity associated with the ventral and medial cell proliferation activity of the otic vesicle, suggesting the existence of other avian Jun family members which have not been cloned yet. However, both c-jun and junD expression seemed to be associated with areas which have been traditionally described of intense cell death (Alvarez and Navascues, 1990).

It has been widely documented that Jun proteins mediate transcriptional stimulation through its interaction with specific DNA sequences (Karin et al., 1997). To assess the functionality of c-jun expression in the inner ear, we explored the DNA binding activity of the c-Jun protein by gel mobility shift assay. For this purpose, a double-stranded oligonucleotide (jun2TRE), which specifically recognises c-Jun-c-Jun homodimers or c-Jun-ATF-2 heterodimers (van Dam et al., 1993), was used as a probe. As shown in Fig. 3, distinct DNA-protein complexes were detected when the radiolabelled probe was incubated with nuclear extracts prepared from otic vesicles (stage 20). The two complexes of slower mobility were completely abolished by the addition of excess unlabelled probe, but not by a 50-fold molar excess of an unrelated oligonucleotide containing a binding site for NF-1. Presence of c-Jun in both complexes was confirmed by supershifting with an antibody to c-Jun. Addition of antibody to the binding reaction resulted in a reduction in the intensity of the retarded bands and the appearance of a weak supershifted band (Fig. 3, closed and open arrowheads, respectively). The intensity of other bands was not affected by the addition of anti-c-Jun antibody. Similar results were obtained when jun1TRE oligonucleotide was used as a probe (data not shown), jun1TRE recognises different AP-1 factors including c-Jun dimers (Ryseck and Bravo, 1991).

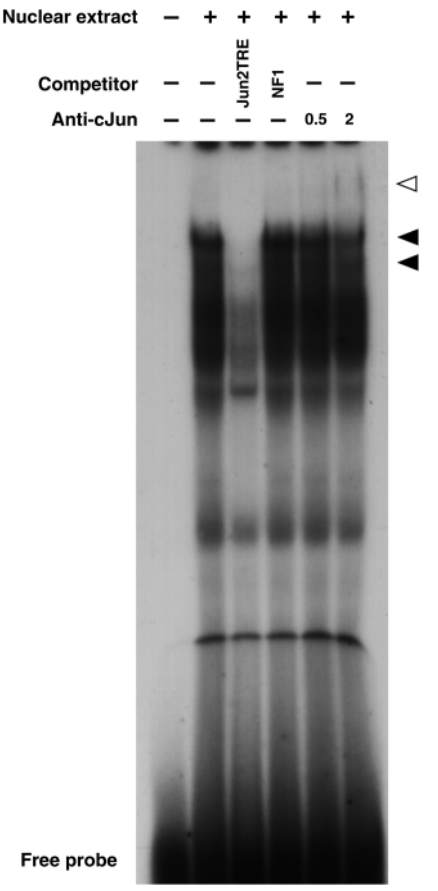

Fig. 3. Gel mobility shift assay of otic vesicle nuclear proteins interacting with an AP-1 site. Labelled double-stranded oligonucleotide jun2TRE was incubated with 10 mg of nuclear extracts prepared from stage 20 otic vesicles. For competition, 50-fold molar excess of unlabelled probe or of unlabelled oligonucleotide containing a consensus NF-1 binding site were used. Prior to the addition of the probe some of the mixtures were incubated for 30 minutes on ice with either 0.5 mg or 2 mg of anti-c-Jun-antibody. The position of the specific protein-DNA complexes are indicated with closed arrowheads. The open arrowhead indicates the supershifted complex. Binding reactions were separated by electrophoresis in a native 5% polyacrylamide gel. DNA-protein complexes are indicated by arrowheads. The experiment shown is representative of at least three independent ones.

Phosphorylation of c-Jun at serine-63 in the developing inner ear

JNKs are members of the MAPKs family whose activation is elicited during apoptosis induced by factors deprivation (Kaplan and Miller, 1997; Leppä et al., 1998) or by NGF (Yoon et al., 1998). JNKs phosphorylate c-Jun at serines 63 and 73 (Karin et al., 1997). This phosphorylation step activates c-Jun

distinctly and leads to an increase in apoptosis. To study the role of c-Jun activation in natural cell death during inner ear development we studied whether c-Jun was phosphorylated by JNKs at the serine-63 residue. The pattern of c-Jun phosphorylation was analysed from stage 14 to 20 by immunohistochemistry with a monoclonal antibody raised against the phosphorylated-c-Jun protein. Fig. 4 shows phospho-c-Jun expression during stages 14 and 19. c-Jun phosphorylation was high at the upper dorsal-caudal edge of the otic pit. In stage 19-20 spots of stained cells were present in the dorsal aspect of the otic vesicle and in the CVG.

Programmed cell death during early otic development

Cells undergoing apoptosis have several morphological characteristics that include membrane blebbing, chromatin condensation, DNA fragmentation and formation of apoptotic bodies that are finally phagocytosed by the surrounding cells. The TUNEL technique takes advantage of DNA fragmentation occurring during apoptosis allowing DNA to be labelled in situ by the terminal deoxynucleotidyl transferase. We used this technique to carefully map cell death areas during the formation of the otic vesicle (Fig. 5). In stage 14, the edges of the otic cup presented a high number of labelled cells. Labelling remained intense in this area until the formation of the otic pore, at the closure of the otic cup to form the otic vesicle (Fig. 5, stages 14 to 18). Latter in development, cell death was detected as scattered cells concentrated in the dorsal-caudal aspect of the otic vesicle around the formation of the endolymphatic duct (Fig. 5, stages 19 and 20). Apoptotic cells were also observed in the ventromedial aspect of otic epithelium, a region from which cells migrate to form the CVG, and also within the CVG (Fig. 5, stage 19 and stage 20). The above description based on the TUNEL technique is in agreement with previous reports using morphological criteria (Alvarez and Navascues, 1990) and complete the description reported by Fekete et al. (1997).

NGF-induction of cell death is associated with c-Jun serine-63-phosphorylation in the otic vesicle

To further study the relationship between c-jun and programmed cell death in the inner ear, we used organotypic cultures of explanted otic vesicles to test the effect of apoptotic and survival factors on c-Jun phosphorylation. NGF induces apoptotic cell death in the dorsal and ventromedial epithelium of the otocyst, and in the CVG (Frago et al., 1998). On the other hand, overexpression of Raf by means of retroviral RCAS vectors suppresses NGF induction of apoptosis and potentiates IGF-I actions as survival factor (Sanz et al., 1999). Otic vesicles were explanted and co-cultured with either CEF or CEF overexpressing Raf for 24 hours, the explants were then cultured for a further period of 16 hours in the absence of serum or presence of factors. Otic vesicles cultured under these conditions exhibited increased apoptosis with respect to otic vesicles cultured in the presence of FBS (Fig. 6A). As shown in Fig. 6, NGF increased phospho-c-Jun staining as evidenced by immunohistochemistry with an antibody specific for phospho-c-Jun C). Both IGF-I (B) and the infection with RCAS/RAF vectors (D) prevented NGF effects on c-Jun phosphorylation. To quantify the effects in cell death resulting from the addition of NGF and NGF plus IGF-I or Raf overexpression, soluble nucleosomes were measured in otic vesicle extracts by using a Cell Death Detection ELISA kit. NGF was a potent inductor of apoptosis and IGF-I was able to suppress NGF-induced apoptosis in otic vesicle organotypic cultures (Table 1). Overexpression of Raf also blocked the response to NGF. Therefore, NGF treatment increased the phosphorylation of c-Jun at serine 63 and cell death, and this effect was prevented by survival factors, in parallel with their ability to suppress cell death.

Table 1. Blockage of NGF-induced cell death

	–	RCAS/RAF
Control	100	100
IGF-I	75±5 (9)*	52±4 (15)*
NGF	159±13 (10)*·‡	87±3 (14)§
NGF + IGF-I	78±5 (12)*·¶	60±5 (4)¶

Otic vesicles were dissected from embryos corresponding to stage 18. Control CEF (–) or infected-CEF (RCAS/RAF) cells were plated in cell culture inserts to a density of 8×10^4 cells/insert and co-cultured with otic vesicle explants in 2% FBS for 24 hours. Explants were cultured for a further period of 16 hours in the absence of FBS (control) or in the presence of 1 nM IGF-I, 4 nM (100 ng/ml) NGF or NGF plus IGF-I. Otic vesicles were homogenised individually and the cell extracts subjected to ELISA determination. NGF and IGF-I data have been previously reported by Sanz et al. (1999). For each experimental condition the absorbance obtained with otic vesicles cultured in the absence of additives was given a value of 100. Results are mean ± s.e.m. of the number of explants tested in at least 3 different experiments. Statistical significance is as follows: *P<0.001 and §P<0.01 vs control, ¶P<0.001 vs NGF, and ‡P<0.001 vs RCAS/RAF plus NGF.

DISCUSSION

A strict balance between cell proliferation and programmed cell death enables the developing organism to control cell number and form. The molecular mechanisms underlying these biological processes are beginning to be understood. A classical route to induce cell proliferation starts after growth factor binding to tyrosine kinase receptors. This is followed by the activation of a cytoplasmic kinase cascade which, in turn, acts on a panel of nuclear targets that results in changes in gene expression. c-fos and c-jun were soon identified as early genes induced by mitogens, and components of the transcription factor AP-1 (Karin et al., 1997). However, more recently it has been described an alternative route that signals apoptosis that involves c-Jun phosphorylation by JNKs (Karin et al., 1997). These findings rise the question of how a cell in a developing organ will take the decision to die or else to survive, on the basis of a given pool of genes encoding proteins that can play opposite roles.

In the present work, we have approached this question by comparing the expression of the avian Jun family members with the pattern of cell death and cell proliferation, during the early development of the inner ear. c-jun and junD are expressed in the dorsal edge of the otic cup (stages 14-17). Expression of both c-jun and junD is very high in the otic pore, but while c-jun expression is restricted to a dorsal narrow band, junD expands to the ventromedial aspects of the otic cup. Thereafter, the temporal pattern of expression of c-jun and junD are also different. In the early otic vesicle (stage 18), the expression of c-jun is maintained while junD is not. Concomitantly, the otic vesicle enters a period of high cell

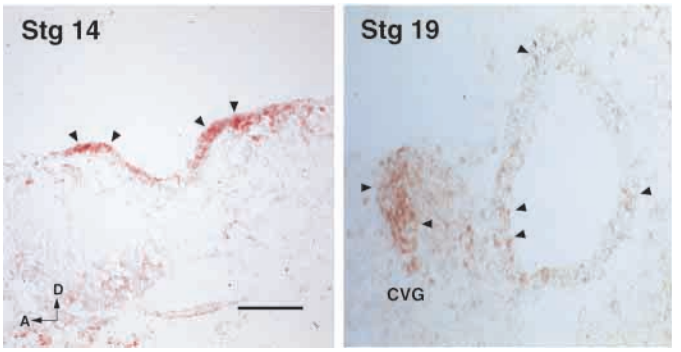

Fig. 4. Phosphorylation of c-Jun at serine-63 in the developing inner ear. Immunohistochemical detection of c-Jun p39 phosphorylated at serine 63 on frozen sections obtained from embryos in stage 14 and 19. At least ten embryos were fixed in 4% paraformaldehyde, cryoprotected in sucrose and sectioned at 20 μm. Immunohystochemistry was performed with an anti-phospho-c-Jun mouse monoclonal antibody that reacts specifically with c-Jun p39 phosphorylated at serine 63. Arrowheads point to the spots of stained cells present in the edges of the otic pit in stage 14, and in the dorsal part of the otic vesicle and CVG in stage 19. Bar, 150 μm. A, anterior; D, dorsal; CVG, cochleovestibular ganglion.

proliferation that is mainly supported by the cells located at its medioventral aspect (De la Pompa, 1995). junD has been associated with inhibition of cell growth (Pfarr et al., 1994). Therefore, the observed down-regulation of junD from potential proliferative areas could be required to allow the burst of cell proliferation in the otic vesicle. Jun proteins form either homodimers or heterodimers with different proteins that include the Fos-related proteins, ATF-2 and CREB. Dimerization takes place through leucine zipper domains and allows binding to DNA sequences, that are present in the target genes (Karin et al., 1997). There are a large number of different combinations of dimers that allow specific transcriptional responses. Fos is homogeneously expressed in the otic vesicle, although with intense levels in the ventral and medial aspects (León et al., 1995b, 1998). Under the assumption that Fos requires of a Jun counterpart to induce cell proliferation, it can be speculated that Fos requires of a yet unknown Jun protein for the induction of AP-1. The central argument rests on the observation that the ventral expression of Fos, where most proliferating activity occurs, is no accompanied by any known Jun member, but it is associated with the detection of Jun protein by means of a highly conserved antibody.

As mentioned above, c-jun expression is high in the dorsal aspect of the otic vesicle and in the CVG from stages 14 to 20, the latest stage studied. Up to stage 17, c-jun expression intersects with that of junD, but then, there is no other Jun counterpart yet described. Therefore, either c-Jun or JunD homodimers or c-Jun/JunD heterodimers are probably the majority forms of AP-1 in the dorsal otic primordium. This is precisely the region where apoptosis occurs most intensely (see below).

Our gel retardation experiments provide the evidence that c-Jun protein in the otic vesicle is effectively translated and binds to specific DNA sequences and, therefore, functionally active. Regarding the signalling mechanism that orchestrate Jun activation, Jun proteins can be activated by phosphorylation

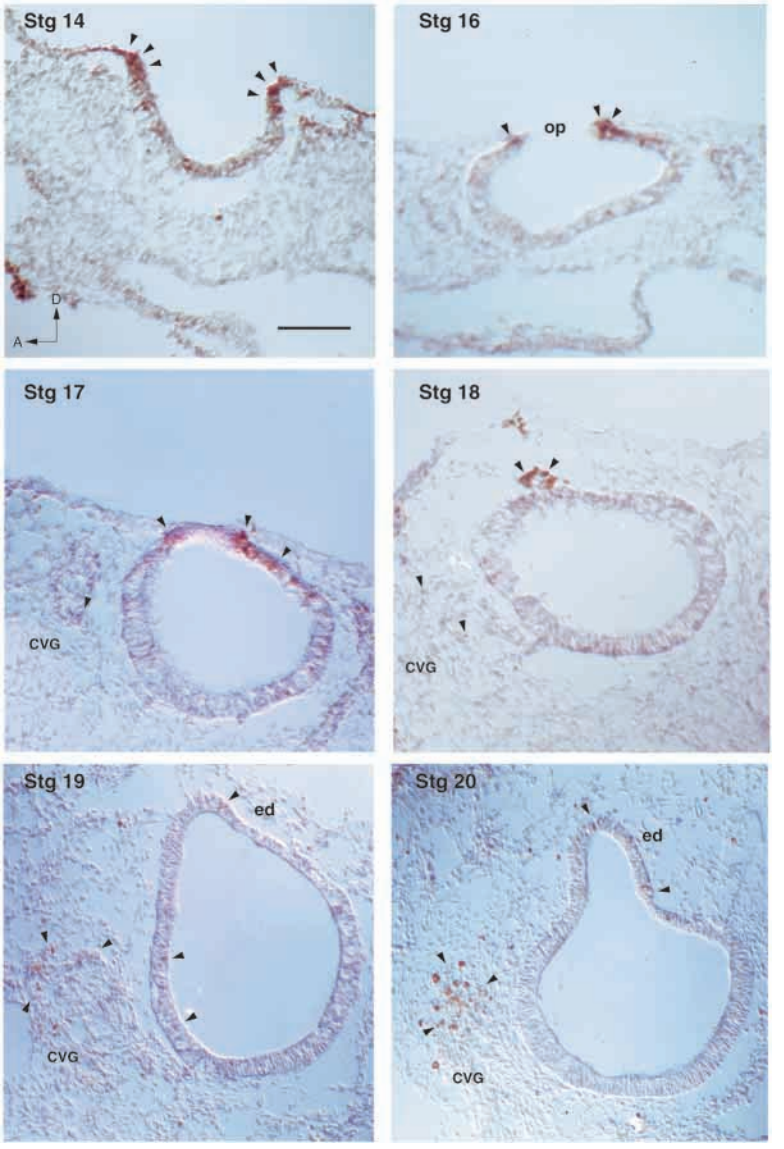

Fig. 5. Pattern of programmed cell death during early otic development. The distribution of apoptotic cells in the otic vesicle was determined by the TUNEL technique on frozen sections as indicated in Materials and Methods. Chicken embryos between stages 14 to 20 were dissected, fixed in 4% paraformaldehyde, cryoprotected in sucrose and sectioned at 20 μm. The fragmented DNA was labelled by the terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labelling reaction. The incorporated fluorescein was detected by anti-fluorescein antibody conjugated with horse-radish peroxidase (Converter-POD). After substrate reaction, the apoptotic nuclei were analyses under light microscope. Arrowheads point to the apoptotic cells. The experiment shown is representative of 3 performed with at least 6 embryos per stage. Bar, 100 μm. A, anterior; D, dorsal; op, otic pit; CVG, cochleovestibular ganglion; ed, endolymphatic duct.

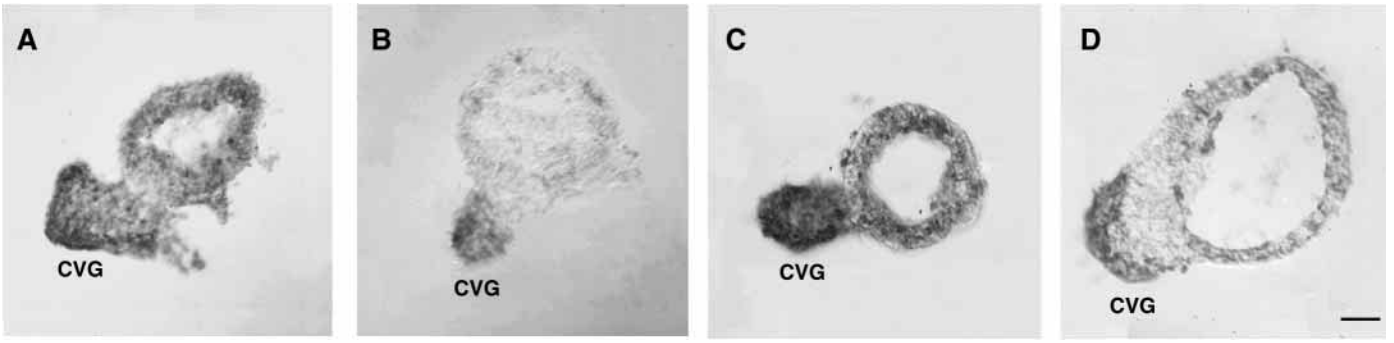

Fig. 6. NGF-induction of cell death is associated with c-Jun phosphorylation in cultured otic vesicles. Otic vesicles were dissected in stage 18 and co-cultured with either uninfected CEF (A,B,C) or CEF overexpressing Raf (D) in 2% FBS for 24 hours. Explants were after cultured for 16 hours in the absence of FBS (A), or with 1 nM IGF-I plus 4 nM NGF (B) or 4 nM NGF (C). (D) Otic vesicles co-cultured with CEF overexpressing c-Raf in the presence of 4 nM NGF and processed as above. Explanted otic vesicles were fixed in 4% paraformaldehyde, cryoprotected in sucrose and sectioned at 20 μm. Immunohystochemistry was performed with an anti-phospho-c-Jun mouse monoclonal antibody. The specimens shown are representative of at least seven otic vesicles per condition isolated and processed in two different days. Bar, 100 μm. CVG, cochleovestibular ganglion.

through two distinct pathways (Karin et al., 1997). Survival factors induce the activation of a subgroup of MAPKs, the extracellular signal regulated kinases (ERKs), which induce c-jun expression and stimulation of cell proliferation. On the other hand, serum-deprivation or NGF binding to its p75 receptor activate another set of MAPK kinases, the JNKs, which phosphorylate c-Jun at the serine residues 63 and 73 leading to apoptosis (Kaplan and Miller, 1997; Karin et al., 1997). JunD is phosphorylated less efficiently than c-Jun by JNKs while JunB is not phosphorylated in response to JNKs activation (Karin et al., 1997). The information is still incomplete, but it points to the idea that specific phosphorylation of Jun may specify the functional role of Jun proteins. Our analysis of c-Jun phosphorylation at serine-63 indicates that the JNKs pathway is operating in the same areas where c-Jun RNA transcripts are detected by in situ hybridisation.

To further understand the role of Jun in the otic vesicle, it was important to test how tight was the relationship between Jun expression, JNK-dependent Jun phosphorylation and cell death. Our results with TUNEL analysis show that Jun expression and JNK-dependent phosphorylation coincide with those areas of cell death. Areas of cell death concentrate at edges of the otic cup and later in the otic pore and the dorsal otic vesicle around the formation of the endolymphatic duct. Also the CVG shows areas of intense cell death. This pattern corresponds well with previous reports based on morphological criteria (Alvarez and Navascues, 1990). Apoptosis could be contributing to the formation of the otic vesicle by facilitating the migration of the upper dorsal cells and hence allowing a closer contact between both borders and also it could be a mechanism to allow the separation of the otic vesicle from the ectoderm. A similar mechanism has been proposed for other embryonic structures, including the neural tube (Weil et al., 1997). In this connection, JNKs signalling has been implicated in morphogenesis and JunD mutant embryos fail to close the dorsal ectoderm in *Drosophila* (Noselli, 1998).

A variety of extracellular factors, intracellular signal transducers and transcription factors interact in the otic vesicle to regulate cell proliferation and cell death. NGF and IGF-I have opposite actions in the otic vesicle. NGF induces apoptosis after binding to the low affinity p75 receptor in restricted areas of the otic vesicle and the CVG, and this effect is completely abolished by overexpression of Raf. NGF induces the generation of ceramide that by itself is able to induce apoptosis in the otic vesicle (Frago et al., 1998). Ceramide has been reported to initiate apoptosis through the JNK cascade (Westwick et al., 1995; Verheij et al., 1996). On the contrary, IGF-I promotes cell growth and survival in the epithelium of the otic vesicle and in the CVG. IGF-I activates the Raf/MAPKs pathway and increases concomitantly the levels of Jun proteins and of the cell cycle PCNA (León et al., 1998; Sanz et al., 1999). In this regard, it is worth noting that apoptosis mediated by c-Jun is not associated with cell cycle progression indicating that the role of c-Jun in apoptosis might be distinct from its function in cell proliferation (Karin et al., 1997). In the present work, we were able to show that NGF induces JNKs-mediated phosphorylation of c-Jun along with cell death. These effects are both blocked when the MAPKs pathway is activated by either treatment with IGF-I or Raf overexpression. This situation is analogous to those reported for pheochromocytoma PC12 cells (Kaplan and Miller, 1997; Leppä et al., 1998) and for mature oligodendrocytes (Yoon et al., 1998).

In summary, we show: (i) the differential patterns of expression of Jun proteins during inner ear organogenesis; (ii) the presence of functional c-Jun in the otic vesicle and its activation by phosphorylation; (iii) that these events are closely associated with programmed cell death in the otic primordium; and, (iv) that both c-Jun phosphorylation and apoptosis are induced by NGF and blocked by IGF-I or Raf overexpression in cultured otic vesicles. Taken together these results indicate that during inner ear organogenesis the distinct expression and activation of Jun proteins are central to achieve the specific cellular responses required for normal development to progress.

L.A. and Y.L. held a CSIC research contract, C.S. was supported by Ministerio de Educación y Cultura and S.C. by the Comunidad de Madrid. This work was supported by grants from Dirección General de Investigación, Ciencia y Tecnología (PM96-0075 and PB95-0086), Europharma (Boehringer Ingelheim Inc.) and Junta de Castilla y León

to F.G and I. V.-N. We also thank Antonio Fernández and Javier Pérez for photographic work and illustrations, respectively.

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