## ROLE OF RAF KINASES DURING INNER EAR DEVELOPMENT

Carmen SANZ[#], Yolanda LEON[#], Mercedes GARCIA-GIL[*] and Isabel VARELA-NIETO[#]
[#]*Instituto de Investigaciones Biomédicas, CSIC, Madrid (Spain) and*
[*] *Dept. of Physiology and Biochemistry, University of Pisa (Italy)*

The vertebrate inner ear develops from the embryonic otic vesicle. This is a transient structure that undergoes a distinct period of cell proliferation that precedes the differentiation of the various cell types that populate the adult ear. The development of the chicken inner ear has been reproduced *in vitro* and established as a model system to investigate factors and molecular mechanisms that regulate cell growth (Leon et al., 1995).

The *raf* family of proto-oncogenes has been highly conserved during evolution. The c-*raf* gene encodes a cytoplasmic serine/threonine protein kinase involved in mitogenic signal transduction from the plasma membrane to the nucleus (Daum et al., 1994). In mammals there are three known active members of the Raf family of kinases: c-Raf, A-Raf and B-Raf. The expression of the B-Raf isoform is restricted to the nervous system and testes (Storm et al.,1990). Homologues of c-*raf* and B-*raf* have been cloned in avian cells and named c-*mil* and c-*Rmil* respectively. Raf proteins are essential for growth and development (Daum et al., 1994). For instance, they are necessary for vulval development in *Caenorhabditis elegans* and for R7 photoreceptor formation in *Drosophila melanogaster*. Little is known about the role of Raf kinases in vertebrate development. Only recently, has the involvement of c-Raf in mesoderm induction in *Xenopus laevis* been demonstrated (MacNicol et al., 1993).

We have studied the expression of c-mil and c-Rmil proteins during the early stages of development of the chicken inner ear. Fig. 1 shows their levels of expression along the different stages of development. Levels of c-mil do not vary, possibly because the regulation is at the level of the kinase activity. However, the expression of c-Rmil has two peaks. The first one at stage 16-17 when the neuroblasts that will form the cochleo-vestibular ganglia (CVG) migrate from the ventromedial area of the otic vesicle. The second peak occurs at stage 20, coinciding with maximal cell proliferation of the CVG (Hemond and Morest, 1991).

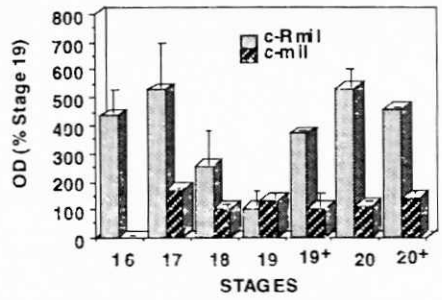


***FIGURE 1. Expression of c-mil and c-Rmil in the otic vesicle.***

Otic vesicles were dissected from chicken embryos of the stages indicated (Hamburger and Hamilton, 1951), lysed in sample buffer and subjected to SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes (Immobilon P, NEN). Membranes were incubated with anti-human c-Raf (UBI) or anti-human B-Raf antibodies, followed by ECL detection (Dupont, NEN) and quantified by densitometry. Data are expressed as percentage of Raf-protein content in stage 19, calculated as mean±SEM of at least 3 experiments.

To study further the role of the *raf* family of proto-oncogenes during inner ear development we have ectopically overexpressed c-*raf* and a cDNA coding for a dominant-negative protein, Raf-C4B, which has a deletion in the kinase domain (Bruder et al., 1992). Both cDNAs were cloned in a replication competent retroviral avian vector, RCAS (Petropoulos and Hughes,1991) and introduced into chicken embryo fibroblasts (CEF) by calcium phosphate precipitation (Cepko, 1992). We have studied the consequences of the overexpression of these genes in CEF by testing mitogen activated protein kinase (MAPK) degree of phosphorylation, as an index of Raf activity. Table 1 shows that cells overexpressing c-*raf* presented higher levels of MAPK phosphorylation in response to epidermal growth factor (EGF), foetal calf serum (FCS) or phorbol esters (PMA) than cells infected with RCAS-AP or uninfected. These results suggest that c-Raf levels are limiting in this model system for the transduction of the extracellular stimuli.

**TABLE 1**

**MAPK phosphorylation levels in CEF**

| | CEF | | | |
|---|---|---|---|---|
| ADDITIONS | UNINFECTED | RCAS-AP | PCSM10 | RCAS-C4B |
| NONE | 1.0 | 1.0 | 1.0 | 1.0 |
| FCS 10% | 1.3 | 2.0 | 6.3 | 0.6 |
| PMA 100 nM | 6.3 | 4.1 | 10.2 | 3.0 |
| EGF 20 nM | 2.7 | 3.2 | 5.2 | 0.8 |

CEF overexpressing the cDNAs coding for the human alkaline phosphatase (AP), c-Raf (PCSM-10) or C4B were treated for 10 min at 37C with either FCS , PMA or EGF at the doses indicated. Then, cells were lysed and the levels of phosphorylated MAPK were evaluated by Western blot analysis as above. The monoclonal anti-phosphorylated MAPK antibody was from BioLabs.

Otic vesicle explants were then co-cultured with CEF producing virus and hence were infected. In the presence of 2% FCS otic vesicles overexpressing c-*raf* had a size increase of 1.65±0.1 fold (n=3) compared to control explants. Similar results were obtained when the levels of proliferating cell nuclear antigen (PCNA) were measured in explants cultured under different conditions. Neither overexpression of the dominant negative mutant nor infection with a control virus bearing the human alkaline phosphatase gene had effect on cell growth.

These results indicate that avian retroviruses are a useful tool to modify the levels of key signalling proteins during embryonic development, and suggest that the Raf family of kinases may have an important role in the control of cellular proliferation and differentiation during the ontogenesis of the inner ear.

### *Acknowledgements*

C.S. , Y.L. and M. G.-G. are supported by Ministerio de Educación y Ciencia (Spain) and the Human Capital and Mobility Programme of the EU, respectively. This work was supported by grants from DGICYT, Europharma (Boehringer Ingelheim group) and Comunidad de Madrid. We wish to thank Dr. Ulf R. Rapp for providing us with the C4B and c-*raf* cDNAs and the anti B-Raf antibody.

### *References*

Bruder, J.T., Heidecker, G. and Rapp, U.R. (1992). Serum-, TPA-, and Ras-induced expression from AP-1/Ets-driven promoters requires Raf-1 kinase. *Genes Develop.* **6**, 545-556.

Cepko, C.L. (1992). Transduction of genes using retrovirus vectors. In "Current Protocols in Molecular Biology", (F.M. Ausubel et al. Eds.) Units 9.10-9.14. Greene and Wiley- Interscience. New York.

Daum, G., Eisenmann-Tappe, I., Fries H-W., Troppmair J. and U.R. Rapp (1994). The ins and outs of Raf kinases. *TIBS* **19**, 474-479.

Hamburger V and Hamilton, H.L. (1951). A series or normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49-92.

Hemond, S.G. and Morest, D.K. (1991). Ganglion formation from the otic placode and the otic crest in the chick embryo: mitosis, migration, and the basal lamina. *Anat. Embryol.* **184**, 1-13.

Leon, Y., Vázquez, E., Sanz, C., Vega, J.A., Mato, J.M., Giráldez, F., Represa, J. and Varela-Nieto, I. (1995). Insulin-like growth factor-I regulates cell proliferation in the developing inner ear, activating glycosyl-phosphatidylinositol hydrolysis and fos expression. *Endocrinology.* **136**, 3494-3503.

MacNicol, A.M., Muslin, A.J. and Williams, L.T. (1993). Raf-1 kinase is essential for early Xenopus development and mediates the induction of mesoderm by FGF. *Cell.* **73**, 571-583.

Petropoulos, C.J. and Hughes, S.H. (1991). Replication-competent retrovirus vectors for the transfer and expression of gene cassettes in avian cells. *J. Virol.* **65**, 3728-3737.

Storm, S.M., Cleveland, J.L. and Rapp, U.R. (1990). Expression of raf family proto-oncogenes in normal mouse tissues. *Oncogene.* **5**, 345-351.