Instituto Juan March
de Estudios e Investigaciones

27 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Ras, Differentiation and Development

Organized by

J. Downward, E. Santos and D. Martín-Zanca

A. Balmain
G. Bollag
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INDEX

PROGRAMME

RAS, DIFFERENTIATION AND DEVELOPMENT

MONDAY, April 25th

Registration

Opening. E. Santos

MOLECULAR MECHANISMS OF RAS FUNCTION

I. Mechanistic and Regulatory Aspects
Chairperson: E. Santos

D.R. Lowy - Regulation of Ras in Mammalian Cells.

G. Bollag - Identification of a Novel Ras Regulator: A Putative Nucleotide Dissociation Specificity Factor.

J. Settleman - Connecting the Ras and Rho Signalling Pathways.

J. Downward - Regulation and Function of Ras Proteins in Cell Growth and Differentiation.

II. Participation in Signalling Pathways
Chairperson: D. Martín-Zanca

L.A. Feig - Regulation of Ras Activation.

C.J.Marshall - Ras, Raf and MAP Kinase.

J.C. Lacal - Signal Transduction of Ras Oncogenes Through Activation of PLD.

TUESDAY, April 26th

RAS IN TISSUE/ORGAN DIFFERENTIATION

III. Ras and Tissue Differentiation/Dedifferentiation
Chairperson: A. Pellicer

A. Balmain - Transgenic Approaches to the Analysis of *Ras* Gene Function in Multistage Carcinogenesis in the Mouse.

E. Santos - Ras and Insulin Signalling Pathways in Adipocytic Differentiation of 3T3 L1 Cells: Dissociation between RAF-1 Kinase and the MAPK/RSK Cascade.

E.J. Taparowsky - Signalling Pathways Regulating Skeletal Muscle Differentiation: Pivotal Role of Activated Ras p21 in Modulating the Transcriptional Activation of Muscle-Specific Genes.

M. Perucho - Somatic Genomic Instability at Microsatellites: A Persistent and Early Event in Cancer of the Recessive Mutator Phenotype.

IV. Neural Tissues
Chairperson: J.C. Lacal

F. Giraldez - Developmental Regulation of Auditory Neurones by Neurotrophic Factors.

J. Martín-Pérez - Association of c-Src with Prolactin Receptors.

D. Martín-Zanca - The *TRK* Family of Neurotrophin Receptors. Structure and Expression.

Presentations of Selected Posters:

W. Chen - Characterization of a Promiscuous GTPase-Activating Protein that Has a Bcr-Related Domain and a Pleckstrin Homology Motif from *Caenorhabditis elegans*.

L. Luo - Drosophila Small GTPase *Drac1* Is Involved in Axon Outgrowth and Myoblast Fusion.

J.C. Stone - Genetic Analysis of Ras Effector Function.

E.V. Avvedimento - Ras Oncogene Interferes with Cyclic AMP Transduction Pathway.

WEDNESDAY, April 27th

RAS IN DEVELOPMENT

V. Ras in Developmental Model Organisms
Chairperson: M. Perucho

K. Kornfeld - Ras-Mediated Signal Transduction During Vulval Development in *C. Elegans*.

F. Karim - Ras and Eye Development in *Drosophila*.

A. Pellicer - N-Ras Gene Expression in Transgenic Mice.

Presentations of Selected Posters:

A. Aranda - Control of Genes Involved in PC12 Cells Proliferation and Differentiation by Nerve Growth Factor and Ras.

M. Lorenzo - Permanent Brown Adipocyte Cell Lines Transfected with Normal and Mutated Ras Genes Maintained a Differentiated Phenotype.

S.J. Morley - Phosphorylation of eIF-4α is Increased During Meiotic Maturation of *Xenopus* Oocytes.

E.M. Fitzgerald - Effect of p21-Ras Antibody (Y13-259) on Electrophysiology of Voltage-Dependent Calcium Channels in Cultured Rat Dorsal Root Ganglion Cells.

VI. Ras in Yeast Models
Chairperson: J. Moscat

M. Yamamoto - Ras Pathways in the Fission Yeast *Schizosaccharomyces Pombe*.

J. Erickson - Biochemical and Genetic Analysis of the Yeast BUD5 Gene Product; its Role as an Exchange Factor for Rsr1 and Ras2.

A. Levitzki - Ras Signalling in *Saccharomyces Cerevisiae*.

J. Downward - General Discussion and Concluding Remarks.

INTRODUCTION

E. Santos

The physical distance between the cell surface and the nucleus is tiny, yet it encompasses the major biological question of how the cells of higher organisms respond to the myriad different signals they receive from the environment. This is a question of major relevance, not only for understanding physiological cell processes, but also to understand the basis of pathological processes such as cancer.

For most of the last decade it was increasingly evident that Ras proteins were essential intermediates for the internalization of signals initiated at the cell surface. However, finding a detailed mechanism through which Ras proteins perform such a function was a rather elusive task. In fact, only last summer the confluence of several separate, distinct fields of Ras research culminated in the description of the so called "Ras pathway" that allowed, for the first time, to trace a signaling pathway from membrane to the nucleus without any obvious gap.

This piecing-together of an apparently universal signaling pathway, with Ras at the core of the process, will probably stand out as one of the milestones in biology during the 90's. It allows, for the first time, to start explaining the mechanism through which many of the signals that direct nucleated cells to divide and differentiate are channelled.

From the scientific point of view it has been intellectually rewarding observing how, as more and more insight was gained into the workings of Ras proteins in specific, evolutionarily separate systems, the apparent complexity of the initial descriptive observations was being progressively replaced by an elegant, simplified, picture of the Ras signaling pathway, which seems to be present, with slight variations, in every eukaryotic organism from yeast to man.

This workshop is precisely dedicated to the study and analysis of specific areas of Ras research that have recently contributed greatly to our current understanding of Ras function. The initial presentations are devoted to mechanistic and regulatory aspects of Ras participation in signaling systems. The biochemical mechanism of Ras activation in signaling pathways initiated by NGF, insulin, or other growth factors is analyzed by various speakers. Functional interactions of Ras proteins with regulatory molecules such as those controling GTPase activity (GAP, NF1) or guanine nucleotide exchange (GDS, GDI) are also discussed in detail.

Another group of presentations is centered around differentiation and development model systems where *ras* gene products have been shown to play important functional roles. Specific areas of discussion include functional studies of *ras* genes in budding (*Saccharomyces cerevisiae*) and fission (*Schizosaccharomyces pombe*) yeast, *Caenorahabditis elegans* vulva development, *Drosophila melanogaster* eye development, muscle differentiation, PC 12 neuronal differentiation, and epidermal, adipocytic and thyroid cell lineages. While some of these systems are only amenable to biochemical analysis, others are susceptible to elegant genetic manipulation which is not usually available in most higher organisms. On looking at all these different model systems, it is striking to note how Ras proteins may be used for a bewildering variety of purposes along evolution, yet they use very similar signaling mechanisms in all cases. Thus, Ras proteins are known to be involved in sensing nutrient conditions and controlling mitotic cycles in budding yeast while they control processes of sexual differentiation in fission yeast. *Drosophila* Ras proteins control development of the complex eye, and in the worm

C. elegans they control proper vulva development. Finally, in mammalian cells, Ras proteins may either stimulate or block proliferation and/or differentiation depending on the cell type involved. The striking observation is that, from yeast to worm to fruitfly or to mammalian cells, the Ras proteins are sitting squarely in the middle of signaling pathways that are arranged in remarkably similar ways, thus confirming broadly the universality of the Ras signaling process.

In its most simplified form, the Ras pathway first involves ligand-receptor interaction at the cell surface that results in formation of the intracellular, active Ras·GTP complex, which then triggers a cascade of phosphorylations reaching the nucleus and leading to modulation of DNA transcription. Upstream from Ras, activation of the receptor tyrosine kinase activity leads to formation of multimolecular complexes where phosphorylated tyrosine residues and other specific molecular motifs (such as Pro-rich sequences), serve as anchors for other SH2- and SH3- (Src homology domains) containing molecules. Some components of these complexes are known to be either positive (SOS, guanine nucleotide exchange factor) or negative regulators (GAP, gtpase activating protein) of Ras activity. Downstream from activated Ras, cytosolic kinases such as Raf-1 and ERKs (MAP kinase, MAPK; S6 kinase, RSK) appear to play a critical part in this cascade. A direct physical association between Raf and Ras has been demonstrated, and associations of Ras with other molecules are also currently being investigated, raising the possibility of multiple downstream targets for Ras.

In any event, as the summary at the end of this booklet points out, there may be more to Ras function than currently meets the eye. Although most mechanisms reported so far suggest a linear channel from surface to the nucleus through Ras, this may only be part of a much more versatile and branched Ras signaling network. Individual cell types may use preferentially specific parts of that network or may offer specific points of cross-talk with other cell-specific signaling networks. That kind of versatility to integrate external stimuli may explain why Ras proteins produce so many different effects in different cellular settings. For example, Ras proteins control both transient processes, such as changes in cell shape and motility, and less reversible processes such as differentiation, cell division and perhaps even programmed cell death.

MOLECULAR MECHANISMS OF RAS FUNCTION

I. Mechanistic and Regulatory Aspects

Regulation of Ras in Mammalian Cells

Douglas R. Lowy,[1] Jeffrey E. DeClue,[1] Maureen R. Johnson,[1] Sylvia Felzmann,[1] Hui Cen,[1] Alex Papageorge,[1] Lise Leonardsen,[2] and Berthe Willumsen[2]
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We have examined factors that negatively and positively regulate Ras activity in mammalian cells. Ras-GAP (GAP) and neurofibromin, the product of the *NF1* gene, have been identified as major negative regulators of Ras. With the exception of schwann cells, GAP appears to be the principal negative regulator of Ras, via its ability to accelerate the GTPase of Ras (GAP-like activity). Neurofibromin appears to be a primary negative regulator in schwann cells, apparently via its GAP-like activity. *NF1* behaves as a tumor suppressor gene in schwannoma cell lines from patients with type 1 neurofibromatosis. Reduction in the level of neurofibromin in such lines is associated with a concomitant increase in the proportion of GTP•Ras. Analysis of other neural crest-derived tumor cell lines has shown that some melanoma and neuroblastoma cell lines established from tumors occurring in patients without neurofibromatosis contain reduced or undetectable levels of neurofibromin, with associated cytogenetic abnormalities of the *NF1* locus. In contrast to the schwannoma lines, GTP•Ras was appropriately regulated in the melanoma and neuroblastoma lines that were deficient in neurofibromin. These results suggested that a tumor-suppressor function for neurofibromin might be independent of its GAP-like activity. As expected for a tumor suppressor gene, introduction of NF1 in melanoma cell lines that are deficient in neurofibromin inhibited their growth and induced their differentiation. In addition, overexpression of neurofibromin in NIH 3T3 cells was growth inhibitory but did not alter the level of GTP•Ras in the cells. Transformation by v-*ras*, whose protein product is resistant to GTPase stimulation by neurofibromin, was inhibited in a cell line overexpressing neurofibromin, while transformation by v-*raf* was not altered. Thus *NF1* is a tumor suppressor gene that can inhibit Ras-dependent growth by a regulatory mechanism that is independent of neurofibromin's GAP-like activity. In more limited studies, we have examined some characteristics of a Ras-specific guanine nucleotide releasing factor (GRF). The catalytic region of the protein is located at its C-terminus. *GRF* cDNAs that include this domain can induce morphologic transformation of NIH 3T3 cells, with an increase in endogenous GTP•Ras. Cells expressing a full length GRF demonstrate an augmented GTP•Ras response to serum. In vitro analysis of the catalytic region has been shown that induces the release of GDP•Ras more efficiently than that of GTP•Ras. The response to GRF is impaired in some Ras mutants.

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Johnson et al, Mol Cell Biol 14: 641-645, 1994

IDENTIFICATION OF A NOVEL RAS REGULATOR: A PUTATIVE NUCLEOTIDE DISSOCIATION SPECIFICITY FACTOR

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Ras proteins possess intrinsic nucleotide exchange and hydrolysis capabilities which are stimulated by cellular factors (Bourne et al., 1990). On the basis of *in vivo* nucleotide metabolism on H-Ras proteins with mutations at Glutamine-61, we have generated evidence for an additional factor whose properties are predicted to inhibit the dissociation of GDP from the Ras proteins. Specifically, these 61-mutants are insensitive to GTPase activation by the GAP proteins (p120-GAP and neurofibromin). However, these proteins display a wide range of transforming potencies in NIH3T3 focus formation assays (Der et al., 1986). By comparing the Ras•GTP levels of these proteins *in vitro* and *in vivo*, we have correlated the transforming potency directly with Ras•GTP levels. This result supports the surprising conclusion that the mutant Ras proteins exchange and hydrolyze nucleotides *in vivo*. Furthermore, our results suggest that there is an inhibitor of nucleotide dissociation in the NIH3T3 cells, since *in vivo* Ras•GTP levels were lower than *in vitro* levels for all of the mutants. This putative inhibitor would behave as a negative regulator of H-Ras-dependent signalling.

By conventional chromatography, we have purified such a factor from Ras-transformed NIH3T3 cells. This factor is a protein of about 40 kDa which is a potent inhibitor of the catalyzed dissociation of GDP from N-Ras. Partial amino acid sequence from the purified mouse protein was identical to a sequence from the human SET protein. SET was originally discovered as an amino-terminal translocation fusion partner (of the CAN protein) in a patient with acute undifferentiated leukemia (von Lindern et al., 1992). Recombinant SET expressed in *E. coli* behaved *in vitro* just as the purified mouse protein. Further characterization revealed that SET could discriminate between N-Ras and K-Ras: SET inhibits GDP dissociation from N-Ras and H-Ras but enhances the exchange factor-catalyzed GDP dissociation from K-Ras. In *Xenopus* oocyte microinjection experiments, SET enhanced the ability of K-Ras and blocked the ability of N-Ras to induce maturation. We are currently testing the effect of SET on the Ras pathway in mammalian cells. Its properties suggest that the role of SET may be to specify which Ras gene product becomes activated.

Bourne et al., 1990, Nature 348, 125-132.
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Connecting the Ras and Rho Signalling Pathways

Rosemary Foster, Madeleine Brouns, Kathy Barrett, Kang-Quan Hu, H. Asha, Iswar Hariharan, and Jeffrey Settleman. MGH Cancer Center and Harvard Medical School. Charlestown, MA 02129

In our efforts to explore the role of the *ras* GTPases in signal transduction and cellular growth control, we have focused our attention on the function of the *ras* GTPase activating protein (*ras*GAP). We and others have observed that the majority of *ras*GAP enters into a tight complex with a cellular protein, p190, in growth-stimulated cells, suggesting that p190 might play a significant role in *ras*-mediated signal transduction (1-3). We have isolated cDNAs encoding p190, and found 2 distinct domains of sequence similarity to previously described proteins (3). At its amino-terminus, p190 has a group of small sequence motifs found in all of the known guanine nucleotide binding proteins, suggesting that p190 is likely to bind GTP and exhibit GTPase activity. At the carboxyl-terminus of p190 is a region of homology to several proteins that have been found to exhibit GAP activity for the *ras*-related *rho* family GTPases, suggesting that p190 should similarly function as a GAP for the *rho* family GTPases. We have recently demonstrated that p190 does, in fact, function biochemically as a *rho*GAP (4). The *rho* GTPases, which are 30% identical to the *ras* proteins, have been implicated in the regulation of cytoskeleton and cell morphology. Thus, the *ras*GAP:p190 complex establishes a direct physical connection between the *ras* and *rho* GTPase pathways, and raises the possibilty that such a complex may serve as an important regulator for the coupling of *ras* and *rho*-mediated signals. As *ras* proteins are known to play a role in controlling cell proliferation and differentiation, and *rho* proteins have been found to function in aspects of cytoskeletal organization and cell shape, it may be that the connection between these pathways ensures that these activities are appropriately coordinated.

The presence of sequence motifs in p190 that are found in all known GTPases prompted us to look for guanine nucleotide binding and GTPase activity of the purified p190. We have been able to demonstrate that the purified p190 protein is associated with GTP, and that this interaction requires the amino-terminal GTPase-like domain of p190 (5). While p190 lacks a detectable GTP hydrolyzing activity under the conditions tested, we have found a weak GTPase promoting activity in crude cell lysates, suggesting the existence of a cellular GAP for p190. The presence of this domain in p190 is intriguing, and raises the possibility that the nucleotide state of the protein regulates some aspect of its cellular function. A mutated form of p190 that fails to bind nucleotide retains its *ras*GAP binding and *rho*GAP activities, suggesting that GTP binding by p190 is not required for these functions. The sequence of p190 in the GTP-binding domain, which shares structural features with both the *ras*-like small GTPases and the larger G-proteins, suggests that this protein defines a novel class of guanine nucleotide binding proteins.

To begin to address the requirement for p190 in *ras* and *rho*-mediated signalling processes *in vivo* we have initiated efforts to disrupt the function of the endogenous p190 gene by the targeted homologous recombination "knockout" strategy in mouse embryonal stem (ES) cells. To initiate the targeted disruption of the mouse p190 gene, we first isolated the mouse p190 gene from a genomic library. An isolated p190 genomic clone was then restriction mapped and an exon that spans the translational start codon was identified by direct sequencing. This clone was then used to construct a "knockout vector" that contains the neo resistance marker and TK gene. Following electroporation with the targeting vector, clones of cells that were resistant to G418 and ganciclovir were expanded, and DNA from each clone was analysed by southern blotting using a probe that can distinguish between random integration of the vector and true homologous recombination. 3 clones out of 270 that were analysed were found to have undergone homologous recombination with the p190 targeting vector. Thus, cells harboring one normal copy of the p190 gene and one disrupted copy of p190 were identified. We are currently using standard ES technology to establish mice that are homozygously disrupted for the p190 gene in order to determine the requirement for p190 in mouse development and to establish p190-negative cell lines from such

animals. This will enable us to address the requirement for p190 in some of the well documented *ras* and *rho*-mediated signalling pathways.

While much has been learned about the role of the *ras* GTPases in a variety of cellular signalling pathways, relatively little is known about the regulation and function of the *rho* family GTPases. Therefore, we have also undertaken investigations that are intended to define components of the *rho* pathway through a genetic analysis in drosophila. An analogous approach to study the *ras* pathway has resulted in the identification of several genes that encode proteins that play a role in *ras*-mediated signal transduction (6,7). The genetic screen that we have chosen to conduct in drosophila relies upon the highly regular pattern of development of the compound eye. The drosophila eye is composed of approximately 800 units called ommatidia, each of which consists of 8 photoreceptor cells, 4 lens secreting cells and 8 accessory cells. The development of each cell within the ommatidium is regulated by specific signals. Even subtle perturbations of this pattern result in a phenotype which can be readily scored by light microscopy.

To initiate these studies, it was first necessary to isolate *rho* family homologs in drosophila. Using a combination of degenerate PCR and hybridisation methodologies, we were able to isolate complete cDNAs encoding two *rac* homologs and a *rho* homolog from a drosophila cDNA library. The predicted amino acid sequence of these clones revealed that they are highly related to their mammalian counterparts. Thus far, we have focused our attention on the *rho* clone. We have placed this cDNA under the control of a promoter (glass-response) that is specifically expressed in cells of the drosophila eye. By microinjecting this DNA into drosophila embryos, we have now established transgenic flies that express the *rho* transgene. We observed that these flies exhibit a dramatic "rough eye" phenotype which is readily distinguished from that of normal flies, indicating that an increase in the level of *rho* activity has profound consequences for normal eye development. Moreover, the *rho*-induced phenotype is highly dose-dependent. This phenotype will serve as the basis for conducting a genetic screen for components of the *rho* pathway. The severity of this phenotype is expected to be increased or decreased by mutations in the genes that encode such components. Identification of protein components of the *rho* signalling pathway will hopefully lead to a better understanding of how this pathway regulates cytoskeletal organization and cell morphology, and potentially, how the *ras* and *rho* pathways are coordinated.

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REGULATION AND FUNCTION OF RAS PROTEINS IN CELL GROWTH AND DIFFERENTIATION

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Ras proteins are regulated by control of their nucleotide binding state, being active when GTP-bound and inactive when GDP-bound. The Sos guanine nucleotide exchange factors have been shown to stimulate Ras in response to growth factors such as EGF. It is currently not known exactly how complex formation of Sos, Grb2 adaptor protein and EGF receptor leads to activation of the exchange activity of Sos toward Ras. Possible mechanisms involve translocation from cytosol to membrane and/or allosteric regulation, perhaps involving accessory molecules such as Shc. A reconstitution system designed to address these questions will be discussed, and also the use of permeabilised cells to tell which interactions are required for Ras activation.

Cyclic AMP has recently been shown to down regulate the Ras signalling pathway by interfering with the interaction of Ras and Raf. Another down regulation pathway will be discussed, in this case responding to the activation of Ras itself. This involves the transmission of a conformational change from Sos, through the SH3 domains of Grb2, to the SH2 domain of Grb2.

A number of possible effectors acting downstream of Ras have now been identified. While it has been shown that Ras interacts directly with Raf in a GTP dependent manner both *in vitro* and *in vivo*, it has not been possible to show an effect of this binding on the kinase activity of Raf. A number of possible ways in which this interaction could lead to Raf activation in cells are being investigated. In addition the involvement of phosphatidyl inositol 3' kinase in mediating the Ras response will be discussed.

II. Participation in Signalling Pathways

REGULATION OF RAS ACTIVATION

Larry A. Feig, Tufts University School of Medicine, Boston, MA, 02111

Activation of Ras proteins is a key step in the transmission of signals emanating from all receptors studied to date that act through membrane associated tyrosine kinases and some receptors that act through heterotrimeric G proteins. Ras proteins become activated by exchanging GTP for prebound GDP. The rate limiting step, the release of prebound GDP, is catalyzed by a family of proteins known a Guanine Nucleotide Exchange Factors (GEFs). Some GEFs (mSOS-1 and mSOS-2) are expressed ubiquitously, whereas some (Ras-GRF and Vav) have more limited tissue expression patterns. The latter therefore may play a role in tissue specific Ras function.

We have focussed our attention on Ras-GRF, which is expressed preferentially in brain and have been comparing its properties to the ubiquitously expressed SOS proteins. By using in situ hybridization, we have detected a distinct pattern of GRF expression in brain that is developmentally regulated.

SOS couples to tyrosine kinase receptors by its ability to bind the SH3 domains of the adaptor protein, Grb2. The SH2 domains of Grb2, in turn, bind tyrosine phosphorylated residues on activated receptors, such as that for EGF. We have therefore studied GRF interactions with this system and found that unlike, SOS, GRF does not bind Grb2 in vitro or in vivo. Moreover, unlike SOS, GRF cannot be detected in a complex with the EGF receptor upon growth factor addition to cells. These results suggest Ras-GRF is coupled to another signalling system that is used in brain to activate Ras. Experiments will be discussed whose aim is to detect this novel input pathway.

Ras, raf and MAP kinase

CJ Marshall, S Cowley, H Paterson and P Kemp

The signal transduction pathway from receptor tyrosine kinases via p21ras, raf, MAP kinase kinase and MAP kinase is activated by a wide variety of external signals leading to cell proliferation or differentiation. Oncogenic forms of ras or raf will transform NIH3T3 cells and differentiate PC12 cells but it is not clear whether these effects are mediated by activation of the MAP kinases. We have recently identified the residues of MAP kinase kinase-1 (MEK) which are phosphorylated by raf (Alessi et al 1994 EMBO J 13: 1610-1619) this has enabled us to generate constitutively activated mutants of MAP kinase kinase-1 by replacing sites of phosphorylation with acidic amino acids and to generate interfering mutants by substituting alanine residues. These mutants have been used to study whether activation of the MAP kinase pathway is necessary and sufficient for cellular responses. The activated mutants stimulated PC12 cell neuronal differentiation and transformed NIH3T3 cells. The interfering mutants inhibited growth factor induced PC12 differentiation, growth factor stimulation of proliferation and reverted v-Src and Ras transformed cells. These results therefore show that, depending on cellular context, activation of MAP kinase kinase is necessary and sufficient for cell differentiation or proliferation.

Signal transduction of Ras oncogenes through activation of PLD

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Reinitiation of DNA synthesis in quiescent cells is the result of cooperation among multiple signalling pathways (Cantley et al., 1991, Pouyssegur & Seuwen, 1992). Potent mitogens like PDGF or FGF are capable of activating several distinct signal transducing elements. One of the important signals is the rapid activation of the Ser/Thr kinases cascade, involving Raf-1, MEK and MAP kinases (Pelech & Sandhera, 1992). Also, the generation of several lipid-derived intracellular messengers, both at early and at late times of stimulation, seems to play a role in this complex cascade of events that finally leads to mitogenesis (Warden & Friedkin, 1985; Exton, 1990).

Most growth factors studied can induce the activation of both a PI-PLC in fibroblasts as well as a PC-PLD as an early event (less than 10 min). Moreover, activation of the Ser/Thr kinases cascade takes places within this early time window, indicating that some of the most critical triggering signals should be placed at early times of stimulation. However, this seems to be not the only requirement, since growth factors need to be present for at several hours in order to efficiently induce DNA synthesis (Pledger et al., 1977; Pledger et al., 1978).

Phosphatidylcholine (PC) metabolism has been proposed as an essential late event in the regulation of cell growth. This hypothesis is based on the sustained elevated levels of DAG and *P*Cho at late times (after 4 h) of stimulation of quiescent cells by growth factors (Warden & Friedkin, 1985; Exton, 1990; Cuadrado et al, 1993), and the finding of constitutively elevated levels of DAG and *P*Cho in *ras*-transformed cells (Lacal et al., 1987). Two hypothesis have been proposed. On one hand, a specific PC-PLC activity has been suggested as the target for PDGF and *ras*-p21 pathways (Larrodera et al., 1990). Alternatively, generation of *P*Cho by the consecutive activation of a PC-PLD and choline kinase (ChoK) has been proposed to be the critical enzymes involved in this process (Carnero et al, 1994 a, b; Cuadrado et al, 1993).

We present evidence that generation of phosphorylcholine and diacylglycerol is observed at late times of serum stimulation without any significant activation of a phosphatidylcholine-specific phospholipase C (PC-PLC). Instead, a 2-fold, transient activation of PLD activity was observed after addition of serum, while a residual 20% increase over basal, unstimulated levels was observed for several hours after stimulation. By contrast, a 5-fold constitutive activation of the endogenous PLD activity was observed in *ras*-transformed cells.

To further strengthen our conclusion of a lack of involvement of a PC-PLC enzyme, a complete inhibition of *P*Cho production was observed in cells treated with a choline kinase inhibitor, hemicholinium-3, with no effect on the levels of DAG. Finally, the elevated levels of DAG in both normal cells stimulated with serum and in *ras*-transformed cells, were sensitive to a PA-phosphatase inhibitor, propranolol, with no effect on the levels of *P*Cho. Thus, our results indicate that in normal NIH 3T3 cells stimulated by growth factors and in *ras*-transformed cells, generation of *P*Cho and DAG do not depend on the same direct enzymatic activity, and most likely is a consequence of activation of the PLD enzyme, followed by choline kinase and PA-phosphatase. Furthermore, the difference found in PLD activation between normal cell proliferation and constitutive proliferation induced by *ras*, may represent a major significant difference between normal cells and *ras*-transformed cells.

In parallel studies using the *Xenopus laevis* oocytes model, we have shown that microinjection of the three most relevant types of phospholipases thought to be involved in signal transduction cascades (PLA_2, PLC and PLD) are capable of inducing oocyte maturation in a similar way as the oncogenic *ras* proteins. This effect is induced through the generation of their known second messengers (Carnero and Lacal, 1993). We have investigated the activation of cdc2 kinase, MAP kinase and S6 kinase II after microinjection of phospholipases A_2, C and D and have correlated their mechanisms of activation with that of *ras*-p21 proteins in this system. While all phospholipases analyzed were able to activate MPF, only PLD was able to activate MAP kinase and S6 kinase II, a pattern which correlated with the *ras*-p21 signaling pathway. In addition, microinjection of *ras*-p21 induced a rapid generation of PA as a consequence of activation of an endogenous PLD activity. Thus, the biological activity of *ras* oncogenes in fibroblasts and *X. laevis* oocytes may be functionally linked to activation of a PC-PLD enzyme.

We have investigated whether the two lipid metabolites generated after serum stimulation of quiescent cells or *ras* transformation (*P*Cho and DAG) play any role in regulation of cell growth. We have evidence suggesting that *P*Cho may constitute an important new second messenger for fibroblast's cell proliferation, since blockage of ChoK activity by HC-3 treatment completely eliminated DNA synthesis stimulation by several growth factors (Cuadrado et al, 1993). We have investigated the putative functional correlation of *ras* oncogenes with the PKC pathway, through the generation of DAG. While in *Xenopus* oocytes, inhibition of PKC by staurosporine, bisindolmaleimide and either the A or Z-pseudosubstrates, did not affect *ras* function, in NIH3T3 cells, inhibition of the DAG-sensitive PKC isoenzymes had a drastic inhibitory effect on *ras* function. On the other hand,

overexpression of the DAG- and Ca^{2+}-insensitive PKCζ isoenzyme or a kinase-death mutant to as much as 20-40 fold, had no effect on the normal proliferation properties of NIH 3T3 cells under normal conditions. Cells overexpressing PKCz did not grow in low serum, or made colonies in soft agar, nor induce tumors in nude mice. Finally PKCζ overexpression did not affect the activation of the transcription factor NKκB, as suggested by other authors, nor altered the activation of this factor by TNF treatment. Thus, in NIH 3T3 cells, *ras* proteins seem to require a DAG-sensitive PKC isoenzyme for induction of DNA synthesis.

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NF-κB ACTIVATION DURING RAS AND CYTOKINE SIGNALLING

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The ζ isotype of protein kinase C (ζPKC), a distinct PKC unable to bind phorbol esters, is required during NF-κB activation as well as in mitogenic signalling in Xenopus oocytes and mammalian cells. To investigate the mechanism(s) for control of cellular functions by ζPKC, this enzyme was expressed in E.coli as a fusion protein with maltose binding protein (MBP), to allow immobilization on amylose beads to study signalling proteins in extracts of Xenopus oocytes, that might form complex(es) with ζPKC. Evidence for interaction with the NF-κB/IκB pathway was obtained. Thus, MBP-ζPKC but not MBP, bound and activated a potentially novel IκB kinase of around 50 kDa molecular weight able to regulate IκB-α function. Activation of the IκB kinase was dependent on ζPKC enzymatic activity and ATP, suggesting that ζPKC controls, directly or indirectly, the activity of a functionally significant IκB kinase. We also show here that ζPKC associates and activates MKK/MAPK in vitro, suggesting that one of the mechanisms whereby overexpression of ζPKC leads to deregulation of cell growth may be accounted at least in part for by activation of the MKK/MAPK complex. However neither MKK nor MAPK are responsible for the putative IκB phosphorylating activity. These data provide a decisive step towards understanding the functions of ζPKC.

RAS IN TISSUE/ORGAN DIFFERENTIATION

III. Ras and Tissue Differentiation/Dedifferentiation

Transgenic approaches to the analysis of *ras* gene function in multistage carcinogenesis in the mouse.

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Tumour development in the mouse proceeds in a series of well defined stages involving sequential genetic alterations in oncogenes and tumour suppressor genes. Studies in mouse skin have shown that it is possible to derive cell lines which represent these different stages of carcinogenesis and which retain the appropriate genetic and biological characteristics of the *in vivo* tumours. Previous studies have shown that H-*ras* gene mutations play an important role not only in initiation of carcinogenesis, but also in the promotion and progression phases. Benign tumours frequently have numerical alterations of chromosomes 7, on which the H-*ras* gene is located, but also of chromosome 6, which harbours the c-raf-1 gene. Overexpression of each of these genes may play a synergistic role in the early promotion stage. Further alterations in *ras* gene dosage occur at the transition to highly undifferentiated spindle cell carcinomas in which mutant *ras* alleles are frequently homozygous and/or amplified. During this sequence, the effector functions stimulated by *ras* appear to be progressively altered from differentiation or quiescence to proliferation or tumorigenicity. In order to address these questions, we have generated transgenic mice expressing *ras* genes in different sub-populations of cells within the epidermis to investigate the effects an differentiation and proliferation. Previous results have shown that expression of a mutant human *ras* gene in suprabasal keratinocytes under the control of a keratin 10 promoter induced hyperkeratosis and papilloma formation. Expression of the same gene from a truncated basal keratin promoter (K5) induced acanthosis and formation of both keratoacanthomas and papillomas. In contrast to the results observed with the K10-*ras* transgenic mice, some of the benign tumours in K5-*ras* mice progressed to malignancy.

Additional experiments have been carried out using animals in which the p53 gene has been inactivated by homologous recombination. The use of these transgenic and knockout animals has allowed us to demonstrate a causal role for H-*ras* and p53 in tumour initiation and tumour progression respectively. The cross-breeding of these and other transgenic or knockout animals should enable us to identify crucial roles played by a number of oncogenes, growth factors or tumour suppressor genes in multistage carcinogenesis.

RAS AND INSULIN SIGNALING PATHWAYS IN ADIPOCYTIC DIFFERENTIATION OF 3T3 L1 CELLS: DISSOCIATION BETWEEN RAF-1 KINASE AND THE MAPK/RSK CASCADE.

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We showed previously that insulin-induced differentiation of 3T3 L1 cells can be mimicked by expression of transfected *ras* oncogenes (1). Exposure of 3T3 L1 cells to insulin results in formation of the active Ras·GTP complex without GAP tyrosine phosphorylation, and expression of dominant negative Ras mutants inhibits insulin-induced differentiation, demonstrating that p21Ras is mediating insulin signaling in these mammalian cells (1,2).

Expression of *ras* oncogenes in 3T3 L1 adipocytes caused also marked elevations of both GLUT1 and GLUT4 glucose transporter proteins in plasma membranes relative to intracellular membranes, and insulin had no further effect (3). These results suggest that activated Ras proteins may also mediate some of insulin's acute actions, such as regulation of hexose uptake and glucose transporter redistribution to the cell surface.

Insulin treatment of untransfected 3T3 L1 cells quickly induced activation of a cytosolic 42 kDa mitogen-activated protein kinase (MAPK) and a 90 kDa S6 kinase (RSK). The activation of these cytosolic extracellular signal-regulated kinases (ERK) was also mimicked by Ras expression (in the absence of insulin) in the same cells transfected with inducible *ras* oncogenes(2,4). Furthermore, insulin-induced activation of MAPK and RSK could be blocked by expression of a transfected, inducible dominant negative Ras mutant (N17). These results indicate that Ras proteins are obligatory intermediates in the activation of cytosolic ERK kinases by insulin (4).

Insulin treatment of 3T3 L1 cells or expression of transfected *ras* oncogenes resulted also in hyperphosphorylation of cellular Raf-1. Insulin-induced Raf hyperphosphorylation was inhibited by expression of an inducible, dominant negative Ras mutant (N17). We also showed that expression of transfected *raf* oncogenes induces adipocytic differentiation, as detected by expression of the specific adipocytic marker aP2. In addition, insulin-induced differentiation was significantly blocked by expression of a dominant negative *raf* mutant. Interestingly however, the expression of transfected *raf* oncogenes did not induce MAPK or RSK activation, and the insulin-induced activation of these kinases was not blocked by expression of transfected dominant negative *raf* mutants (4). These results are consistent with Raf kinases acting downstream of Ras, but not upstream of MAPK and RSK in insulin signaling pathways leading to 3T3 L1 differentiation.

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Signaling Pathways Regulating Skeletal Muscle Differentiation: Pivotal Role of Activated Ras p21 in Modulating the Transcriptional Activation of Muscle-Specific Genes. *Yanfeng Kong, Xueyan Wang, Tushar B. Vaidya, Stephen F. Konieczny and Elizabeth J. Taparowsky.* Department of Biological Sciences, Purdue University, West Lafayette, IN USA 47907-1392.

The formation of skeletal muscle *in vivo* involves the determination of pluri- or multi-potential stem cells to proliferating precursors of the skeletal muscle lineage (*i.e.*, myoblasts) and the subsequent differentiation of determined myoblasts into multi-nucleate skeletal myofibers. The isolation of a family of muscle-specific basic helix-loop-helix transcription factors (*i.e.*, the muscle regulatory factors, or MRFs) has led to a number of *in vivo* and *in vitro* experiments demonstrating that MRF activity orchestrates the genetic events required for both the determination and the differentiation of skeletal muscle cells (reviewed by Olson. Mol. Endocrin.7:1369-1378.1993). Studies from our laboratory have suggested a central role for the MRFs in regulating skeletal muscle differentiation since several recognized inhibitors of myogenesis in culture - namely, FGF-2, TGF-β1 and oncogenic Ras p21 - function by blocking the ability of the MRFs to activate the transcription of muscle-specific genes (Konieczny *et al.* Oncogene *4*:473-481. 1989; Vaidya *et al.* Mol. Cell. Biol. *9*:3576-3579. 1989; Hardy *et al.* Mol. Cell. Biol. *13*: 5943-5956. 1993). In investigating the molecular mechanism(s) underlying inhibition of MRF activity, we have explored the potential role of a.) differential protein phosphorylation, b.) DNA binding efficiency and c.) oligomerization partner preference. Interestingly, our analysis of MRF functions in cells treated with FGF-2 and TGF-β1 are not in complete agreement with those we have obtained with Ras p21 and, in fact, suggest that the endpoint of Ras p21 inhibition of skeletal muscle differentiation is not the MRFs, but rather one or more MRF accessory proteins. Our current efforts are focused on defining the Ras p21 target molecules in muscle cells, on determining how the target molecules influence MRF activity and on continuing to investigate if Ras p21 plays a role in FGF-2 or TGF-β1 signaling during myogenesis.

SOMATIC GENOMIC INSTABILITY AT MICROSATELLITES: A PERSISTENT AND EARLY EVENT IN CANCER OF THE RECESSIVE MUTATOR PHENOTYPE.

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That spontaneous errors in DNA replication may be substantial in transformation was proposed in an attempt to explain the chromosomal alterations of cancer cells. A replication defective factor could generate an enhanced error rate in the clonal variants arising during tumor progression. However, increased mutation rate in tumor cells has not been demonstrated despite intensive efforts.

The reproducible and quantitative amplification of many genomic sequences in a single and simple experiment by the Arbitrarily Primed PCR or AP-PCR (Welsh & McClelland, NAR *18*, 7213, 1990), is a powerful tool for the unbiased analysis of the genetic alterations accompanying malignancy. We have shown that both allelic losses and gains can be easily detected and characterized by AP-PCR genomic fingerprinting which thus provides an alternative molecular approach for cancer cytogenetics (Peinado *et al*, PNAS *89*, 10065, 1992).

We have also found that about 12% (16 of 137) of colorectal carcinomas have sustained somatic mutations in numbers that in some cases can be estimated by extrapolation to reach the million (Ionov *et al*, Nature, *363*, 558, 1993). These mutations are deletions of one or a few nucleotides in monotonic runs of dA:dT base pairs, including the poly(A) tails of repetitive *Alu* sequences, and also deletions and insertions in other simple repeats such as the polymorphic CA repeats and other microsatellite sequences. These ubiquitous somatic mutations (USM) at simple repeated sequences (SRS) are clustered in tumors with distinctive genotypic and phenotypic features and ubiquitously present in all neoplastic regions of synchronous tumors from the same patient, including adenomas. We concluded that these mutations unveil a distinct molecular pathway for cancer corresponding to the "cancer as a mutator phenotype" hypothesis (Loeb, Cancer Res. *51*, 3074, 1991). Because these clonal mutations were present in all neoplastic areas of each of synchronous tumors from the same patient, including adenomas, we inferred that they are the consequence of a "*mutator mutation*" (a mutation in a gene coding for a replication or repair factor that results in decreased replication fidelity) which plays an ultimate causal role in tumorigenesis. We also suggested that USM in SRS are due to slippage

by strand misaligment (Streisinger *et al*, Cold Spring Harbor Symp. quant. Biol. *31*, 77, 1966) and that the mutator mutation may have a hereditary predisposition, with tumors with USM in SRS overlapping with those of the hereditary nonpolyposis colorectal cancer (HNPCC) Lynch Syndrome.

These hypotheses have been confirmed and loci linked to HNPCC families have been identified (Thibodeau *et al*, Science, *260*, 816, 1993; Aaltonen *et al*, Science, *260*, 812, 1993) and shown to be involved in mismatch repair. Both somatic and germline mutations in these recently cloned genes have been shown to segregate with HNPCC (Fishel *et al*, Cell, *75*, 1027, 1993; Leach *et al*, Cell, *75*, 1215, 1993; Bronner *et al*, Nature, *368*, 258, 1994; Papadopoulos *et al*, Science, *263*, 1625, 1994).

We have determined the mutation rates of endogenous microsatellite sequences in several colon tumor cell lines. At least two levels of instability have been defined, with some cell lines showing mutation rates about 10 fold higher than normal cells or tumor cells without microsatellite instability, while other tumor cell lines exhibit mutation rates at least two orders of magnitude higher. We also have shown that microsatellite instability is a very early event in tumor development, and that this genomic instability persists in tumor cells of the mutator phenotype, which continue to accumulate mutations during tumorigenesis as consecutive slippage events of a single or a few repeated units (Shibata *et al*, Nature Genetics, *6*, 273, 1994). We conclude from these results that this is the same mechanism underlying the repeat expansions of triplet hereditary diseases and that the same defect in replication fidelity observed in cancer of the mutator phenotype may also contribute to the non Mendelian anticipation of these diseases.

Somatic cell hybrid experiments indicate that the genomic instability at microsatellite sequences is recesive because hybrids between cell lines with and without instability completely regain the stability of replication of these repeated sequences. Introduction of a wild type chromosome 2 does not restore the replication fidelity in some cell lines with low instability. Hybrids between cell lines with low and high instability also regain the fidelity of replication for simple repeated sequences (Casares *et al*, in preparation). These experiments, in concert with repair assays with cell free extracts, have defined at least four functional complementation groups for mismatch repair involved in human cancer (Umar *et al*, J. B. Chem, 1994, in press).

IV. Neural Tissues

DEVELOPMENTAL REGULATION OF AUDITORY NEURONES BY NEUROTROPHIC FACTORS.

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The cochlear ganglion contains the primary afferent neurones of the vertebrate auditory system. They receive their synaptic input from the sensory receptor cells of the cochlea and project to the cochlear nuclear complex of the brainstem. Cochlear neurones originate from the otic vesicle and start to differentiate after performing their terminal mitosis. Innervation proceeds in parallel with hair-cell maturation to form the first synaptic contacts and then mature synapsis (Whitehead & Morest, 1985). Morphological events correlate with the functional expression of ionic-currents studied with the 'patch-clamp' technique. Potassium-currents are expressed very early, coinciding with the start of innervation. Then, Na^+ and K^+-current densities increase throughout development. Slowly-inactivating K^+-currents dominate early and intermediate stages, and a delayed-rectifier type of non-inactivating K^+-current predominates in mature neurones. A rapidly inactivating potassium current similar to the A-current, I_A, is distinctly expressed in the more advanced developmental stages (Valverde et al., 1991). Calcium-currents are just visible when first synaptic contacts are established and fully develop in parallel with maturation of synaptic contacts.

The NGF-family of neurotrophins (NGF, BDNF/NT-4, NT-3) exert distinct effects on developing cochlear neurones, including stimulation of cell-proliferation during early stages and survival and neuritogenesis during innervation periods (Represa et al, 1991; Avila et al., 1993). Early mitogenic effects appear to be coupled to low-affinity binding and to the hydrolysis of a membrane glycosyl-phosphatidyl-inositol. Differentiation effects, however, require high-affinity binding sites and are more sensitive to BDNF and NT-3 than to NGF. Biological effects overlap with the differential expression of both low (p^{75}) and high-affinity receptors. The later belong to the *trk* proto-oncogene receptor family which expression is restricted to neurones within the cochlear ganglion and regulated throughout development. Experiments also show that otic-conditioned media are able to mimic some of the effects produced by neurotrophins, like stimulation of neurite outgrowth. They can induce also the appearance of other phenotypic characteristics such as the expression of Ca^{2+}-currents in cultured cochlear neurones. Similar studies have been performed on vestibular neurones which are associated with the sense of equilibrium and postural reflexes, and that have a similar embryological origin. These and other results indicate that the NGF-family of neurotrophins are good candidates to regulate the development of the inner ear. Specificity of responses is achieved by spatial and temporal restriction of factors and receptors and their intracellular messenger mechanisms, resulting in regulation of cell-division or differentiation of receptor and neuronal networks.

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Association of c-Src with Prolactin Receptors

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The Src family of proto-oncogenes had been implicated in early steps of cellular growth. The association of these protein tyrosine kinases with the cytosolic domains of a number of receptors provoke their activation which is an essential step in the mechanisms of signal transduction by these receptor ligands (1).

The Prolactin receptors (PRLR) is constituted by a family of surface transmembrane proteins generated by alternative splicing, that are widely distributed in animal tissues, and are members of the larger family of cytokines receptors (2). As for other members of this family, the PRLRs have no identifiable motifs in the cytoplasmic regions that might indicate the signal transducing mechanisms. Being the liver an important physiological target for prolactin, we have found that PRLR immune complexes from hepatocytes contain a cytosolic tyrosine kinase. We have identified in isolated hepatocytes the tyrosine kinase as the $pp60^{c\text{-}src}$. Incubation of hepatocytes with PRL induces the $pp60^{c\text{-}src}$ association with PRLR and the tyrosine phosphorylation of this kinase. Furthermore, PRL stimulates the expression of c-fos, c-jun, and c-Src (3).

When chicken embryo fibroblasts (CEF) were transfected with the long form of the PRL-R, we also detected the association of this receptor with c-Src. Prolactin not only increased this interaction but also promotes protein tyrosine phosphorylation (4). This last model system will allow us to study the implications of this association in the mechanisms of signal transduction by this hormone. All these findings support the concept that the $pp60^{c\text{-}src}$ tyrosine kinase is involved in the early steps of PRL intracellular signaling.

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THE *TRK* FAMILY OF NEUROTROPHIN RECEPTORS. STRUCTURE AND EXPRESSION.

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The *Trk* family of transmembrane tyrosine kinases encodes high affinity receptors for the nerve growth factor family of neurotrophic factors. Thus, NGF is the preferred ligand for *Trk*A, which can also bind NT3 and NT4/NT5, albeit less efficiently. BDNF and NT4/NT5 are the preferred ligands for *Trk*B, whereas NT3 is a secondary ligand for this receptor. Finally, NT3 is the only known ligand for *Trk*C. The degree of promiscuity of neurotrophin binding to *Trk* receptors is reduced in PC-12 cells and primary neurons as compared to fibroblasts, suggesting that in neuronal cells there might be accessory molecules restricting the ability of *Trk* receptors to interact with their non-preferred ligands (1,2,3). However, crosstalk between neurotrophins and their receptors may have physiological relevance during nervous system development, *in vivo*.

Structural features of the extracellular ligand binding domain of the *Trk* molecules define a novel class of tyrosine kinase receptors characterized for a specific combination of sequence motifs, conserved among all the *Trk* family members. These include, three tandem leucine rich repeats, probably involved in protein-protein interactions, and perhaps in receptor dimerization; and two immunoglobulin-like C2 type repeats similar to those found in cell adhesion molecules, suggesting that, in addition to binding neurotophins, *Trk* receptors could be involved in cell-cell or cell-matrix interactions (4,5). Another striking particularity of the *trk* gene family is that it encodes a number of variant receptor forms as a result of alternative splicing of their mRNAs. There are two forms of *Trk*A, one of which contains a 6 amino-acid insert in the extracellular domain between the second IgC2 repeat and the transmembrane region, which is encoded for by a neuronal specific alternatively spliced exon.

*trk*B and *trk*C encode full length as well as truncated receptors lacking the TK catalytic domain. Whereas the former are almost exclusively expressed in neurons, the latter are also expressed in non-neuronal cells. It has been suggested that these truncated forms are involved in transporting, concentrating or presenting ligands to the complete receptors. In cells coexpressing both forms, the kinase-less receptors can act as dominant negative "mutants" by forming heterodimers with the full length ones. Finally, in the case of *Trk*C, additional forms exist that contain variable length amino-acid inserts in the TK catalytic domain. This insert-containing receptors show increased TK activity in response to NT-3 binding but fail to mediate proliferative and differentiation effects in fibroblasts or PC-12 cells, respectively. This lack of activity correlates with their inability to phosphorylate PLCγ and to activate PI3 kinase. *Trk*C is the only known receptor tyrosine kinase with different catalytic domain isoforms, and different substrate specificities (6).

NGF is the prototype of a family of neurotrophic factors essential for the survival, differentiation and maintenance of specific populations of neurons in developing an adult nervous system (7,8). Since the neurotrophins act mostly on postmitotic neurons and elicit essentially non-mitogenic responses, it was somehow surprising to find that they would signal through classical tyrosine kinase receptors, structurally similar to those used by mitogenic growth factors. Nonetheless, this similarity clearly extends to the mechanism of *Trk* receptor activation as well as their signaling pathways. NGF binding to *Trk*A induces receptor dimerization, activates its kinase and results in the autophosphorylation of a number of tyrosine residues. Specific phosphotyrosine residues of *trk*A have been recently identified as binding sites for SH2-containing substrates such as PLCγ, which binds to Y785; PI3 kinase, to Y751; SHC, to Y490; and, perhaps, ERK1 (9,10).

The NGF signaling pathway known to date is similar to that of mitogenic growth factors, with *Ras* playing a pivotal role connecting the activated receptor and its substrates to the activation of downstream serine/threonine kinases such as *Raf*-1, MEK, MAPK and RSK. Yet, NGF induces differentiation, not mitogenesis. What is, then, the basis for this specific response?. By using PC-12 cells which can differentiate in response to NGF or FGF, and proliferate in response to EGF, a nuclear protein, SNT, has been identified whose phosphorylation in tyrosine is preferentially stimulated by differentiation and not by mitogenic factors.

Phosphorylation of SNT, although perhaps nsecessary, is not sufficient to induce differentiation, since mutant form of *Trk*A unable to associate to SHC and to PLCγ, and devoid of differentiation activity, still induce SNT phosphorylation. In addition, dominant negative mutants of *Ras*, which efficiently block NGF-induced differentiation, do not affect NGF-stimulated SNT phosphorylation, indicating that this protein acts upstream or in a parallel pathway to that of *Ras* (10,11). Therefore, it is possible that differentiation results from the stimulation of a combination of signaling pathways, some of which are common to mitogenic stimulation and some are differentiation specific.

*trk*A expressing neurons closely match those that are NGF-dependent for survival *in vivo*. Thus, *trk*A mRNA is found in a subset of neural crest-derived sensory neurons of the trigeminal, superior, jugular, and dorsal root ganglia (DRG), as well as in sympathetic neurons. In the CNS, *trk*A is expressed only in cholinergic neurons of the basal forebrain. This precise pattern of expression provided the first clues that led to the identification of *Trk*A as the NGF receptor, and suggested that *Trk*A could mediate NGF actions *in vivo*. This has now been demonstrated by generating mutant mice carrying homozygous deletions of the *trk*A gene. *trk*A "knock-out" mice are insensitive to heat and pain, and show extensive loss of small-diameter neurons in the trigeminal and DRG ganglia, which are known to convey nociception and thermoreception stimuli. They are also devoid of sympathetic neurons, and have reduced numbers of cholinergic projections to the hippocampus and cortex. This phenotype is entirely consistent with the pattern of TrkA expression and with the known neuronal specificity of NGF effects in vivo, thus proving that the neurotrophic actions of NGF are mediated trough the *Trk*A receptor (12).

*trk*A expression marks selected groups of neurons for survival in response to NGF. This factor rescues them from death during development and induces their differentiation. In addition, NGF has a continuing role in survival and maintenance of a particular functional phenotype in the adult nervous system. *trk*A expression may be part of the developmental program of these neurons or perhaps contributes actively to define it. In either case, understanding the mechanisms that control *trk*A expression will undoubtedly help define, at the molecular level, some of the events leading to the acquisition of a specific neuronal phenotype.

Our goal is to dissect the *trk*A promoter to identify the regulatory sequences acting in *cis*, as well as the transcription factors acting in *trans*, which are responsible for the exquisite pattern of *trk*A expression *in vivo*. To this end, we have isolated the mouse *trk*A gene in a series of overlapping genomic clones. Detailed analysis of the 5' most region of the gene, including nucleotide sequencing of some 4 kbp around the first exon, has allowed us to identify the start of transcription and to detect promoter activity using *trk*A-reporter fusions.
A combination of primer extension and S1 nuclease protection analysis, using RNA from mouse embryo DRG, allowed us to map the *trk*A transcription start site some 70 nucleotides upstream of the ATG translation initiation codon. This gives a short leader to the *trk*A mRNA as compared to that of other *trk* genes.
Detailed analysis of the nucleotide sequence around the first *trk*A exon reveals a very high GC content and a ratio of observed to expected CG frequency, consistent with the region being a CpG island. CpG islands are regions of the genome very rich in the dinucleotide CpG that are not methylated.CpG islands are usually associated to the 5' region of housekeeping genes (13). Analysis of the methylation status of some of these CG dinucleotides, using appropriate restriction enzymes, has shown that indeed they are unmethylated, even in DNA from tissues not expressing *trk*A. Thus, *trk*A belongs to a growing group of genes that, in spite of showing a very specific pattern of expression, have CpG islands at their 5' ends.
In order to detect promoter activity we have generated transcriptional fusions between *trk*A DNA fragments and reporter genes such as CAT and *lac*Z. By transfecting them into PC-12 as well as NIH3T3 cells we have been able to define a region of some 2 kbp, 5' of the ATG, which exhibits transcription activity in PC-12 cells but not in fibroblasts.
Primer extension analysis using total RNA from PC-12 cells transfected with the *trk*A-*lac*Z fusion has show faithful transcription initiation in this cells. This results indicate that a relatively short DNA fragment contains the *trk*A promoter and some "neuronal"-specific regulatory sequences, which we are in the process of characterizing.

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Characterization of a Promiscuous GTPase-activating Protein That Has a Bcr-Related Domain and a Pleckstrin Homology Motif from *Caenorhabditis elegans*

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Human Breakpoint cluster region (*Bcr*) gene product is a member of a group of GTPase-activating proteins which act exclusively on members of the Ras-related Rho subfamily. A complementary DNA was isolated from *Caenorhabditis elegans* that encoded a polypeptide of 1438 amino acid residues, CeGAP, which contains a domain with sequence similarity to the COOH-terminal segment (GTPase-activating region) of Bcr and other known GTPase-activating proteins of the Rho subfamily. It also contains a 'pleckstrin homology' motif, present in many signaling proteins including GTPase-activating proteins and nucleotide-exchange factors. The Bcr-like domain of CeGAP exhibited activity not only on members of the *C. elegans* and human Rho subfamily, but surprisingly also on *C. elegans* Ras protein (let-60), human Ras and Rab3A. CeGAP is therefore the first GTPase-activating protein acting on Ras-related proteins across different subfamilies. Together with the presence of the pleckstrin homology motif, our finding suggests a central and integrative role for CeGAP in a signalling pathway common to Ras and related proteins.

DROSOPHILA SMALL GTPASE *Drac1* IS INVOLVED IN AXON OUTGROWTH AND MYOBLAST FUSION

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The small GTPases of the rac/rho/CDC42 subfamily are implicated in actin cytoskeleton-membrane interaction in mammalian cells and budding yeast. We have cloned Drosophila homologs of rac and CDC42, *Drac1* and *Dcdc42*. They share 70% sequence identity and both are highly expressed in mesoderm and the nervous system during muscle and neuronal differentiation, respectively. We expressed putative constitutively active and dominant negative Drac1 proteins in these tissues. When expressed in neurons, Drac1 mutant proteins cause axon outgrowth defects in the peripheral neurons without affecting dendrites. When expressed in muscle precursors, they cause complete failure of, or abnormality in, myoblast fusion. Expressions of analogous mutant Dcdc42 proteins cause qualitatively distinct morphological defects.

Genetic Analysis of ras Effector Function

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The function of *ras* has been probed using genetic methods. Mutations in the *ras* effector region that uncouple effector function and GAP interaction have been sought to test the hypothesis that GAPs serve as regulatory molecules, not effectors. The influence of effector domain mutations on the coupling of *ras* and Raf is also being explored.

Mutations in the conserved portion of the *ras* effector domain that have effects on the intrinsic biochemical properties of the *ras* protein have also been sought. These studies may indicate that the evolutionary conservation of the effector domain, as originally defined, reflects conservation of functions other than effector signalling.

To define the *ras* effector in fibroblast transformation, extragenic suppressors of non-transforming *v-H-ras* effector domain mutations have been isolated. One somatic mutation is heterozygous for a *c-raf1* substitution. Additionally, this cell clone is altered in some other cellular element that influences the phosphorylation state of Raf. Current experiments are aimed at defining the kinases and phophatases that modify Raf in mutant and wildtype cells.

RAS ONCOGENE INTERFERES WITH CYCLIC AMP TRANSDUCTION PATHWAY

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Thyroid cells like many endocrine cells are dependent on cAMP for growth and differentiation. Specifically, thyrotropin (TSH) activates adenylyl cyclase via a G_s-coupled receptor. cAMP dissociates protein kinase A and the catalytic subunit of the enzyme (cPKA) migrates into the nucleus, where it phosphorylates several transacting factors.

By using a thyroid cell line transformed with a temperature-sensitive variant of Ki-Ras we have analyzed the early steps following the activation of the oncogene.Ki-*Ras* oncogene blocks the expression of thyroid specific genes and dedifferentiates thyroid cells[1,2,3]. One important mediator of Ras is PKC(a), which is stabilized by Ki-*Ras* (protected by TPA induced degradation) and inhibits PKA nuclear accumulation. Therefore, the dedifferentiation of thyroid cells is dependent on the loss of nuclear PKA[3].

By immunofluorescence, immunoprecipitation, and Western analysis with specific antibodies to the regulatory subunits of PKA we have found that: 1. PKAII anchored *via RIIb* on the Golgi apparatus is the source of nuclear cPKA; 2. v-Ras activation or PKC stimulation leads to delocation of typeII PKA (3-12 hours) from the Golgi to the perinuclear membrane; after 1-2 days from the initial *Ras* activation the enzyme diffuses in the cytoplasm, where is degraded. In chronically transformed cells type II PKA (IIb) is lost and the specific transcriptional response to cAMP is repressed.

The location of RIIb and PKAII is cell cycle regulated and parallels changes in the phosphorylation status of the PKA b regulatory subunit. The migration of RII from Golgi to the perinucleus and cytosol accompanies the onset of S-phase. Conversely forced delocalization of this particular form of Golgi kinase (PKAII b) accelerates by 12 hours the entry in S phase of thyroid cells.

Taken toghether these data suggest that 1. location and type of PKA enzyme are essential elements for the transmission of cAMP signal into the nucleus and 2. *Ras* by changing the location and the composition of PKA enzyme alters the flux of cAMP signals to the nucleus.

RAS IN DEVELOPMENT

V. Ras in Developmental Model Organisms

Ras-mediated signal transduction during vulval development in *C. elegans*.
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The nematode *Caenorhabditis elegans* contains a specialized hypodermal structure, the vulva, that forms a passage between the gonad and the environment used for egg-laying and sperm entry. Six cells, P3.p - P8.p, constitute the vulval equivalence group and have the potential to generate vulval tissue. During wild-type development only three of these cells, P5.p, P6.p and P7.p, adopt vulval fates; these three cells generate the 22 descendants that form the vulva. The other three cells, P3.p, P4.p, and P8.p, each generate two descendants that contribute to the general body hypodermis.

Signals have been identified that affect these cell fate decisions [1]. The gonadal anchor cell signals nearby Pn.p cells to adopt vulval cell fates; if the anchor cell is absent, all six cells in the equivalence group adopt hypodermal fates. A signal from the surrounding hyp7 hypodermal syncytium inhibits cells from adopting vulval fates; in the absence of this signal, all six cell in the equivalence group adopt vulval cell fates.

A large number of genes have been identified that affect vulval development, including some that affect these signaling events [2]. Five genes have been described that are required for vulval induction and are similar to genes important for signaling in vertebrate cells. *lin-3* encodes a protein that is similar to the signaling protein EGF and appears to be the signal secreted by the anchor cell [3]. *let-23* encodes a protein similar to the EGF receptor *trans*-membrane tyrosine kinase [4], *sem-5* encodes a protein that contains one SH2 and two SH3 domains similar to the human adapter protein *Grb-2* [5], *let-60* encodes a protein similar to the GTPase *ras* [6], and *lin-45* encodes a protein similar to the serine/threonine kinase *raf* [7]. Loss-of-function mutations in these genes cause all cells in the vulval equivalence group to adopt hypodermal fates, so this pathway is required for vulval induction.

An activating, codon 13 mutation in *let-60 ras* causes all six cells in the vulval equivalence group to adopt vulval fates, resulting in a multivulva (Muv) phenotype [8]. To identify additional genes involved in vulval development, particularly genes that act downstream of Ras, we screened for mutations that suppress this Muv phenotype. Forty-three independent mutations were identified that define 22 complementation groups. We named these genes *suppressor of activated ras* (*sar*). Three genes appear to be required for signal transduction in vulval development because mutants display the same spectrum of phenotypes displayed by *let-23* and *let-60 ras* mutants. One group corresponds to the previously identified *lin-45 raf* gene, while two others, *sar-1* (7 alleles) and *sar-2* (6 alleles) are new genes. We are trying to clone these two genes by using their genetic map positions and the well characterized *C. elegans* physical map. In collaboration with M. Lackner, L. Miller and S. Kim (Stanford University), we have shown that another

complementation group, containing the single allele *n2521*, corresponds to a *C. elegans* homolog of MAP kinase and defines the gene *mpk-1*. Thus, a *C. elegans* MAP kinase homolog acts downstream of Ras and plays an important role in Ras signaling [9].

Mutations in the *lin-1* gene cause a Muv phenotype. Double mutants defective in *lin-1* and any of the signaling genes described above also display the Muv phenotype. Thus, *lin-1* appears to act downstream of these signaling genes. *lin-1* was cloned, and it encodes a protein with a region similar to the DNA-binding ETS domain found in a variety of transcription factors.

We have identified 14 genes that negatively regulate vulval induction and appear to be involved in signaling from the hyp7 hypodermal syncytium [10,11]. These genes can be divided into two classes, A and B, such that double mutants carrying a mutation in any class A gene and any class B gene display a Muv phenotype. However, double mutants containing two class A mutations or two class B mutation appear wild-type. Thus, the genes in class A and class B appear to identify two redundant processes that negatively regulate vulval induction. These genes are termed synthetic multivulva genes. We have molecularly characterized four of these genes: *lin-9*, *lin-15a*, *lin-15b*, and *lin-36*. All four appear to encode novel proteins. In addition, genetic mosaic analysis demonstrated that the *lin-36* gene is likely to be required in the cells of the vulval equivalence group and not in the signaling cells.

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RAS AND EYE DEVELOPMENT IN *DROSOPHILA*

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During development of the *Drosophila* compound eye, cell fate specification of the R7 photoreceptor requires a signal through the Sevenless receptor tyrosine kinase (1). Several genes, including *drk*, *Sos*, *Gap1*, *Ras1*, *Draf* (Raf), *rolled* (MAPK), *yan* and *sina*, have been shown to act downstream of *sevenless* in this pathway (2-8). Expression of constitutively active Ras1 under control of the *sevenless* enhancer/promoter (*sev-Ras1^{V12}*) bypasses the requirement for Sevenless-RTK activation and transforms some non-neuronal precursor cells into supernumerary R7 cells, resulting in roughening of the eye exterior (9). The number of ectopic R7 cells produced is dose dependent; flies bearing two copies of *sev-Ras1^{V12}* have rougher eyes than those carrying only one copy. This correlation between the severity of eye roughness and the strength of signalling in the pathway has been used as the basis for a genetic screen to identify dominant modifiers of Ras1^{V12}. In this screen, a two-fold reduction in the dose of certain genes that act downstream of Ras1 is predicted to alter the rough eye phenotype of *sev-Ras1^{V12}*. We have used this sensitized system to screen ~850,000 progeny and have identified several hundred dominant enhancers and dominant suppressors of *sev-Ras1^{V12}*. In addition to genes known to act downstream of Ras1 [e.g., *Draf* (Raf), *Dsor1* (MAPKK), *rolled* (MAPK), and *yan*], we have identified several genes that encode new components in the the pathway. Two genes that act downstream of Ras1, *yan* and *pointed*, both encode ETS DNA-binding proteins but have opposite effects on R7 photoreceptor development. Loss-of-function *yan* mutants are dominant enhancers of *sev-Ras1^{V12}*, while loss-of-function *pointed* mutants are dominant suppressors. Transcriptional activation of a reporter gene by the Pointed protein appears to be regulated by MAP kinase activity.

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N-RAS GENE EXPRESSION IN TRANSGENIC MICE. R.Mangues,S. Schwartz,I.Seidman and A.Pellicer. Dept.of Pathology,New York Univ.School of Medicine,New York.

One of the intriguing features that characterizes the ras genes is the increasing evidence indicating that they play a role in the signal transduction pathways that can lead the cell into proliferation or differentiation. We have chosen the N-ras gene as the subject of study since we have shown that is involved in mouse lymphomagenesis (1,2) and it can induce PC12 cell differentiation (3). To determine how these two seemingly opposing functions operate in vivo, we have introduced some constructions with the N-ras gene into the mouse germ line.

The initial experiments, where no transgenic mice were born when the N-ras gene was under the control of its own promoter, indicated that overexpression of the gene was deleterious during embryonic development. To determine the timing of this effect, we injected the fertilized eggs either with the N-ras oncogene or with a prokaryotic vector as control. The eggs were cultured in vitro for 6 days and monitored for their development up to hatched blastocyst. The results indicated that, in the group injected with N-ras, there was a higher frequency of arrested embryos which did not reach the stage of hatched blastocyst. In a parallel line of experiments, other N-ras injected eggs were implanted into foster mothers which were sacrificed at day 8 or 10 of pregnancy. The morphologic and molecular analysis revealed a relationship between the embryos who had altered development and the presence of the transgenic N-ras sequences.

To analyze the functions of the N-ras gene in vivo, we substituted its promoter by the MMTV-LTR and we were able to establish transgenic lines containing several copies of the proto-oncogen. These mice develop mammary carcinomas with an incidence of 30% (4,5). To confirm that they only contained the normal gene, DNA from the tumors was sequenced indicating the presence of only the normal N-ras allele. When these animals were treated with the chemical carcinogen MNU there was a significantly decreased latency in the appearance of mammary carcinomas, although the incidence was maintained at 30%. Molecular analysis of those tumors showed that they did not contain any ras mutations indicating that the carcinogen had altered another gene able to cooperate with N-ras in this tumorigenic process. To understand the manner in which the N-ras proto-oncogen predisposes the cells to malignant transformation, we analyzed the expression of this gene in the transgenic animals. We observed that expression of the transgene in normal mammary gland was already elevated several fold with respect to the endogenous N-ras gene. Interestingly, this increased expression is gradual, correlates with mice aging and with

demethylation of a position in the promoter. In tumors there is an additional increase in transgene expression (5). The transgene demethylation in the tumors is significantly greater than in normal contralateral mammary glands or the endogenous N-ras gene. By manipulating the MMTV promoter, we have been able to establish transgenic lines containing the mutationally activated N-ras oncogene. These lines have a high incidence of thymic lymphomas.

All these results support the notion of multiple functions for the ras genes, provide evidence of the importance of their regulation and open the possibility of using these transgenic lines to dissect the ras pathway in vivo.

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CONTROL OF GENES INVOLVED IN PC12 CELLS PROLIFERATION AND DIFFERENTIATION BY NERVE GROWTH FACTOR AND RAS.
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PC12 pheochromocytoma cells exhibit neuronal phenotype upon treatment with NGF or expression of the *ras* oncogene. We have examined their influence on the expression of different genes that accompany PC12 cell differentiation including immediate early genes, the transin gene, and the TGF-β gene. In a subclone of PC12 cells (UR-61) stably transfected with activated N-*ras* under control of a glucocorticoid-inducible promoter (MMTV), addition of dexamethasone (Dx) produced extensive neuronal differentiation. In these cells incubation with NGF increased N10 mRNA levels and Dx totally blocked this response. Incubation of the parental cells with Dx did not affect the response of this gene, thus showing that p21*ras* is the responsible for this inhibition. Other early genes showed a similar response. Expression of the activated oncogene in UR61 cells also led to a significant increase in the levels of transin mRNA. NGF, that induces the activity of this gene in PC12 cells, was not able to increase transin mRNA levels in the presence of activated *ras*. These data agree with the evidence that *ras* is involved in the transduction of the signals generated by NGF and suggests a negative regulatory mechanism acting on signalling pathways normally stimulated by the growth factor when these pathways are maximally activated by the oncogene. Conditioned media from NGF-treated cultures caused colony formation of NRK cells in soft-agar and decreased proliferation of Mv1Lu cells, suggesting an increase in TGF-β expression. Incubation with an anti TGF-β1 antibody totally blocked the inhibitory effect of PC12-conditioned media on ^{3}H-thymidine incorporation in Mv1Lu cells, thus showing that TGF-β1 is the factor released. More than 90% of the factor is in a latent form and is activated by acidification-neutralization treatment of the media. NGF induced a more than 3-fold increase in the concentration of the biologically active factor in the conditioned medium. NGF produced a rapid and dose-dependent increase in TGF-β1 mRNA levels in PC12 cells. Ligands for other receptors with tyrosine kinase activity (IGF-1 or FGF) produced neurite extension and also increased expression of the TGF-β1 gene. Neuronal differentiation induced in UR61 cells by incubation with Dx was accompanied by an increase in TGF-β1 mRNA levels. These cells express nearly normal levels of *trk* mRNA but they do not differentiate in response to NGF. In this subclone NGF did not increase TGF-β1 gene expression. In PC12 cells constitutively expressing a dominant negative *ras*, p21(Asn17), NGF had no effect on TGF-β1 mRNA levels. In contrast, in PC12 cells over-expressing *trk*, the levels of TGF-β1 mRNA were very high even in the absence of NGF and incubation with NGF produced a further increase. Taken together these results suggest a common pathway for receptors with tyrosine kinase activity and the *ras* oncogene which converge in TGF-β expression, and show the requirement of functional p21*ras* for the regulation of the TGF-β1 gene by NGF.

PERMANENT BROWN ADIPOCYTE CELL LINES TRANSFECTED WITH NORMAL AND MUTATED RAS GENES MAINTAINED A DIFFERENTIATED PHENOTYPE.

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IGF-I induced the expression of H-ras by 3-fold at 4 h and 24 h and by 6-fold at 72 h respectively, in fetal brown adipocyte primary cultures. Concurrently, IGF-I increased by 3-fold the percentage of cells in the S, G2 and M phases of the cell cycle, and also induced the mRNA expression of the lipogenic marker malic enzyme as well as the the tissue-specific gene uncoupling protein. Our results suggest that IGF-I is a mitogen also involved in the induction of differentiation-related gene expression, where the activation and/or expression of ras may be a common crucial intermediate (J. Cell Biology 123, 1567-1575, 1993).

Permanent rat fetal brown adipocyte cell lines have already been established by the mean of transfection, of brown adipocyte primary cells, with constitutive gene constructs of SV40 Large T antigen in cooperation with mutated or normal ras genes (Exp. Cell Res. 209, 248-254, 1993). Regardless the observed phenotype, transformed by the expression of mutated H-ras (Lys 12) or flat by normal H-ras (Glyc 12), both clones are quite homogenous as indicated by flow cytometric analysis of several parameters, such as cell size (FSC) and roughness and organuli content (SSC), and showed a proliferative status as indicated by the high percentage of cells in S, G2 and M phases of the cell cycle and the constitutive expression of the nuclear factor PCNA. However, they also maintained a differentiated phenotype, showing a high amount of cytoplasmic lipid content as determined by Nile red fluorescence, and expressing the tissue-specific gene uncoupling protein. The differential effect of serum deprivation on both kind of clones, and well as their response to IGF-I, TGF-β or glucocorticoids will be discussed.

Phosphorylation of eIF-4α is increased during meiotic maturation of Xenopus oocytes.

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Xenopus oocytes can be induced to undergo meiotic maturation in vitro by treatment with either progesterone or insulin. In both cases there is an increase in the overall rate of protein synthesis. Previous work in this laboratory has shown that the increase in protein synthetic activity in response to progesterone is (a) reflected in the activity of cell-free extracts prepared from unstimulated oocytes and mature eggs and (b) exerted at a stage of the initiation pathway at or after the binding of the 40S ribosomal subunit to messenger RNA. We have now analysed events in Xenopus oocytes at different times during the maturation process, and find that maturation is associated with increased phosphate-labelling of the initiation factor eIF-4α. This phosphorylation is a relatively late event, and, in the case of progesterone-induced maturation, appears to coincide with the main increase in the rate of protein synthesis. However, treatment of oocytes with insulin results in a much more rapid rise in the rate of protein synthesis, which precedes detectable phosphorylation of eIF-4α.

Using purified eIF-4α, we have also detected kinase activity towards this factor in extracts prepared from oocytes at a late stage in maturation induced by either hormone. A similar activity was observed in extracts from oocytes microinjected with the active form of $p21^{ras}$, but not with the inactive Gly-12 form (these extracts were kindly provided by Dr. M. Pomerance, U96, INSERM, France). To examine further the upstream signalling events involved in hormone-induced maturation and the associated increases in protein synthesis, we have investigated the effects of rapamycin, which has been shown in a number of cell types to inhibit the activation of the p70 S6 kinase but not the p90 S6 kinase. This agent did not prevent maturation in response to either hormone, nor the activation of protein synthesis. If anything, it increased the hormone-induced labelling of eIF-4α. These data suggest that the early activation of the p70 S6 kinase that has been observed during progesterone-induced maturation of Xenopus oocytes is not necessary for the stimulation of overall protein synthesis or of eIF-4α phosphorylation.

EFFECT OF p21-RAS ANTIBODY (Y13-259) ON ELECTROPHYSIOLOGY OF VOLTAGE-DEPENDENT CALCIUM CHANNELS IN CULTURED RAT DORSAL ROOT GANGLION CELLS.

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Activated p21-ras, in combination with ras-GAP has been found to block activation of atrial K^+ channels by M2 muscarinic receptors, an effect which was suggested to involve an interference with receptor-G-protein coupling (Yatani *et al.*, 1990). Since both M2 and M4 muscarinic receptors are known to inhibit Ca^{2+} channels directly via the pertussis toxin (PTX)-sensitive G-protein, G_o, a similar mechanism involving p21-ras may also be involved in regulation of neuronal Ca^{2+} channels (Jones, 1993; Menon-Johansson *et al.*, 1993). Here we report the results from a study in which the patch-clamp technique (whole cell and perforated patch configurations) has been used to examine the involvement of endogenous p21-ras in regulation of calcium channels from primary cultured rat dorsal root ganglion cells (DRGs).

In preliminary experiments, the presence of the muscarinic agonist carbachol (CARB, 10^{-4} M) in the external medium, was found to cause a 32 ± 4 % (mean ± s.e.m.) inhibition of calcium channel current, I_{Ba}. External addition of the muscarinic antagonist pirenzepine (10^{-7} M), however, reduced inhibition of I_{Ba} to 8 ± 3 %, suggesting the presence in DRGs of either an M1 or M4 receptor. Further, incubation with PTX (230 ng/ml for 15 h) resulted in only 5.1 ± 1 % inhibition of I_{Ba} by CARB. This PTX-sensitivity would tend to suggest the presence of an M4 receptor in rat DRGs. However, the possibility that a PTX-sensitive M2 receptor is present in these cells cannot be ruled out on the basis of these results.

In DRGs microinjected with the p21-ras neutralising antibody (Y13-259, 0.1 mg/ml, 5pl/cell) 1.5 to 2 h prior to experiments, a 43.7 ± 4 % inhibition of I_{Ba} (perforated patches) was observed following application of CARB. This result was not significantly different from control cells injected with 1 mg/ml mouse IgG (36.2 ± 4 %) or from non-injected cells (42.9 ± 4 %). The voltage-dependence of activation of I_{Ba} shifted between 5 - 10 mV in a depolarising direction in all three treatments after CARB application. These shifts in voltage-dependence were not significant either within, or, between treatments. Hence, microinjection of Y13-259 had no effect on I_{Ba} in the presence of the agonist. Interestingly, however, a marked reduction of 36 % in the peak amplitude of the maximum current was observed in Y13-259-injected cells as compared with IgG-injected and non-injected cells (significant at $p < 0.05$). There was no significant effect on the voltage-dependence of activation of the maximum current in antibody-injected cells compared with controls.

In conclusion, neutralisation of endogenous p21-ras by microinjection of the antibody (Y13-259) reduced calcium currents in DRGs but did not affect muscarinic inhibition of these currents. This is consistent with the study of Yatani *et al.*,(1990) where injection of Y13-259 alone had no effect on atrial K^+ channels, but, injection of activated p21-ras in combination with ras-GAP blocked K^+ channels via an M2 receptor. The reduction in peak amplitude of the maximum current in Y13-259-injected cells in the absence of carbachol does, however, suggest that endogenous p21-ras could be involved in regulation of Ca^{2+} channels in DRGs although the effect may not occur via muscarinic receptors. Experiments with activated p21-ras are currently in progress.

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VI. Ras in Yeast Models

Ras pathways in the fission yeast *Schizosaccharomyces pombe*.

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Schizosaccharomyces pombe contains a single *ras* gene, designated *ras1*, that has many of the features conserved in the mammalian *ras* genes. *S. pombe* cells carrying a null mutation in the *ras1* gene are viable and grow at a rate similar to that of wild-type cells. However, haploid cells of both mating types show complete deficiency in mating (sterility) if they are defective in *ras1*. These cells show normal secretion of mating pheromones but fail to respond to them. Homozygous diploid *ras1* mutants sporulate poorly, at a small percentage of the wild-type frequency. In addition to their deficiency in sexual development, *ras1* mutant cells show a curious phenotype that they are short and fat compared with the elongated shape of wild-type cells.

We previously identified three genes that regulate the activity of Ras1 by analyzing *S. pombe* mutants either defective in mating or hypersensitive to the mating pheromones. The *ste6* gene encodes the putative GNRP for Ras1. The *ral2* gene encodes a factor that regulates the activity of Ras1 positively but has no homology with any known protein. The gap1 gene encodes a homolog of mammalian GAP and *Saccharomyces cerevisiae* IRA, and negatively regulates the Ras1 activity.

The *byr1* and *byr2* genes were originally identified as high copy suppressors of the sporulation deficiency of diploid *ras1* mutants. The *byr2*, *byr1* and *spk1* genes encode protein kinases that respectively correspond to vertebrate MAPKKK, MAPKK and MAPK. This protein kinase cascade functions downstream of both Ras1 and the heterotrimeric G protein that couples with the mating pheromone receptors. Thus, Ras1 is likely to modulate the sensitivity of the pheromone recognition pathway according to a certain internal or external signal(s). I will show that expression of *ste6* is regulated by the nutritional conditions via a pathway that involves the cAMP cascade. This implicates that Ras1 can function as a link between the pathway responding to the nutritional conditions and the pathway responding to the mating pheromones. Expression of *ste6* is enhanced by the pheromone signalling, indicating the existence of a positive-feedback loop through which Ras1 stimulates the expression of its own activator.

Besides its role in recognition of the mating pheromones, which has been considerably worked out so far, Ras1 appears to play additional roles in *S. pombe* cells. It is unlikely that the kinase cascade involving Byr2 and Byr1 is the sole target of Ras1, because cells defective in *byr2* or *byr1* are morphologically normal whereas cells defective in *ras1* are dumpy. It has been reported that *ras1* defective cells become spherical and lose the directionality of cell growth once they enter the stationary phase. This may suggest that *S.*

pombe Ras1 covers the same role as RSR1 plays in *S. cerevisiae*. However, a target of Ras1 that determines cell morphology remains totally unidentified. In this connection I will discuss on the possible involvement of the *ral1* gene product in the *ras* pathway for cell morphology. The *ral1* mutant was originally isolated as such that resembles the *ras1* defective mutant in the mating and morphological properties. Sequence analysis of the *ral1* gene showed that it encodes a homolog of the *S. cerevisiae CDC24* gene product, which has been thought to function downstream of RSR1 and to serve as the GNRP for the *rho*-family protein encoded by *CDC42*. Ral1 is homologous also with human Dbl and Bcr.

In addition to the above, our preliminary results suggest that the Ras1 function is necessary for *S. pombe* cells to arrest properly at the G1 phase under poor nutritional conditions. Although the molecular basis for these versatile features of Ras1 is not readily comprehensible at present, further analysis of its versatility will surely lead to a more profound understanding of the cellular activities and the controls over them.

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Biochemical and genetic analysis of the yeast BUD5 gene product; its role as an exchange factor for Rsr1 and Ras2

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In yeast, cell polarity requires several gene products that act to control the selection of a bud site and growth of the bud or mating projection. This genetic pathway provides an opportunity to examine the relationship of Ras to two Ras-related gene products, the yeast Rap1 homolog (RSR1) and the Rho homolog, CDC42. The establishment of bud site selection in yeast is determined, in part, by the action of the small GTP binding protein Rsr1 (Bud1). Rsr1 was originally isolated as a gene, that when over expressed, can suppress the phenotypic effects of the *cdc24* mutation. *RSR1/BUD1* was subsequently isolated in a genetic screen for random budding along with four other genes, *BUD2, 3, 4* and *5*. *BUD2* and *BUD5* were found to be homologous to GTPase-activating proteins (GAP) and guanine nucleotide exchange factors (GEFs), respectively. *BUD5* was also isolated as a multi-copy suppressor of a dominant-negative *RAS2* allele, suggesting that the Ras pathway and the budding pathway may somehow functionally connected. Genetic analysis and DNA homology clearly suggest that Bud5 acts as an exchange factor for Rsr1. However, the exact function of the *BUD5* gene product has not been tested biochemically. Its range of action might include not only Rsr1, but also Ras2 and thus, provide a link between Ras signaling and cell polarity. Therefore, Bud5 was purified as a GST-fusion protein and assayed for exchange factor activity against Rsr1, Ras2 and the mammalian Ras and Ras-related genes K-ras and Rap1a. These results and results obtains with the two-hybrid method will be discussed in light of other data that suggests a more direct role between the *BUD* genes and the G1 cyclins, *CLN1* and *CLN2*.

Ras signaling in Saccharomyces cerevisiae

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Ras 1 and Ras 2 mediate signaling in S.cerevisiae in response to nutrients. Ras signaling requires the presence of Cdc25, but it can be shown that the exchanger is not itself the recipient of the incoming signal, although its presence is essential for signal processing. The second role of the Cdc25 protein is to provide the system with an attenuation device. Upon exposure to glucose, Cdc25 is phosphorylated and the signal is terminated concomitantly to the delocalization of the Cdc25 molecule from the Ras cyclase complex. The possible role of the phosphorylation sites will be discussed.

Another aspect to be discussed is the presence of two Ras proteins: Ras 1 and Ras 2. Data will be presented which suggest a differential role of the two Ras proteins. The relevance of these findings to the mammalian system will be discussed.

POSTERS

RAS-p21 ACTIVATES PHOSPHOLIPASE D AND A2, BUT NOT PHOSPHOLIPASE IN *XENOPUS* OOCYTES

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Xenopus laevis oocytes are a powerful tool for the characterization of signal transduction pathways leading to the induction of DNA synthesis. Since activation of either PLA2, PLC and PLD has been postulated as mediators of *ras* function, we have used the oocyte system to study the putative functional relationship between *ras*-p21 and these phospholipases. Microinjection of either one of the phospholipases induced oocyte maturation, similar to *ras*-p21. Microinjection of *ras*-p21 induced the rapid generation of PA and DAG. However, DAG was sensitive to propranolol, a PA-hydrolase inhibitor, indicating that PLD is the enzyme responsible for generation of PA and DAG. Moreover, microinjection of both PLD or *ras*-p21 induced the late production of lysophosphatidylcholine on a $p42^{MAPK}$-dependent manner, indicating the activation of a PLA2. Inhibition of this enzyme by quinacrine does not inhibit PLD- nor *ras*-induced GVBD suggesting that PLA2 activation is not needed for *ras* or PLD function. Contrary to 3T3 fibroblasts, where *ras*-p21 is functionally dependent for its mitogenic activity on TPA- and staurosporine-sensitive PKC isoforms, in *Xenopus* oocytes, induction of GVBD by *ras*-p21 was independent of PKC. Thus, our results demonstrate the activation of PLD and PLA2 by *ras*-p21 proteins, while no effect on PLC was observed.

Ki-Ras changes the composition of protein kinase cyclic AMP dependent and attenuates nuclear cAMP response.

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Ras oncogene transforms and dedifferentiates thyroid cells. We have previously shown that Ras or PKC stimulation dowregulate nuclear accumulation of the catalytic subunit of PKA (cPKA) (Gen.Dev. 1992 6:1621-1630). Reconstitution experiments using purified nuclei from Ras-transformed cells and cytoplasm from differentiated cells indicate that the inhibition of nuclear cPKA accumulation induced by Ras is at cytoplasmic level.

RNA and protein analysis reveals that in Ras transformed cells there is a complete loss of the PKA Regulatory subunit type IIβ and type IIβ PKA, which is normally anchored at the Golgi system.

The overexpression of RIIβ subunit in Ras trasformed cells,by using expression vectors,restores the nuclear accumulation of cPKA and rectivates a silent thyroglobulin gene.

One early step in Ras activation analyzed by immunofluorescence and Western blot with specific antibodies to PKA components is the delocalization of PKA holoenzyme complex from Golgi-centrosome area to the cytosol.This leads to a rapid degradation of RIIβsubunit and a change in the composition of PKA holoenzyme from an RII to an RI complex. Acute TPA treatment mimicks the Ras effect on the location and downregulation of PKA holoenzyme complexes.

These results support the idea that, in the early step of transformation,v-Ras, viaPKC, delocalizes PKAβ holoenzyme from Golgi-centrosome area and attenuates the nuclear response to cAMP. As consquence the expression of cAMP dependent genes(RIIβ, TSH receptor and thyroglobulin) is inhibited. We suggest that the loss of RIIβ is responsible for the change of PKA composition and ultimately for permanent downregulation of nuclear PKA and of thyroid specific gene expression.

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RAS ONCOGENES TRANSACTIVATE THE MOUSE CYTOMEGALOVIRUS IMMEDIATE EARLY GENES ENHANCER/PROMOTER.

Products of Ras genes strongly stimulate the activity of the reporter gene chloramphenicol acetyl transferase, (CAT), driven by a 1.2 kb fragment of the murine cytomegalovirus immediate early (IE) gene enhancer/promoter (pCMVCAT) in NIH3T3 cells. In cotransfection experiments, a mammalian expression vector containing a v-Ha-ras cDNA, together with pCMVCAT strongly enhance the CAT activity. High pCMVCAT activation was also observed in cell lines carrying stably-transfected ras oncogenes, activated by point mutation or amplification. To define the cis-DNA-elements in the IE MCMV enhancer responsible for transactivation by p21 ras protein, we constructed several plasmids containing the CAT gene under control of MCMV IE enhancer deleted in different regions. The CAT assays demonstrate that sequences responsive to p21 ras protein are scattered throughout the IE enhancer upstream of the transcription start site and are homologous to the binding sites for cellular transcription factors, such as NF_KB, AP1, ATF and SP1. In cotransfection experiments with an activated p21 ras, a six-fold increase of the β-galactosidase activity, of a reporter gene driven by three tandems copies of CRE-sequence (pON407.19R3) was observed. This transactivation can not be prevented by cotransfection of the dominant inhibitory mutant Ha-ras Asn17, suggesting that activated ras protein, rather than normal p21 ras is responsible for it. Eventually, the observed transactivation was accompanied by an increase of nuclear protein binding to a labelled oligonucleotide homologous to the CRE-sequence, as shown in gel-retardation assays. Altogether these results suggest that the CRE-element contributes to transactivation of the MCMV IE enhancer/promoter by ras oncogenes and p21 ras protein may thus be one of the signals that regulating genes transcription during MCMV infection.

DOWNREGULATION OF H-RAS IN TERMINAL DIFFERENTIATION AND GROWTH ARREST OF K562 HUMAN MYELOID LEUKEMIA CELLS

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Human myeloid leukemia K562 cells can be terminally differentiated *in vitro* towards erythroid lineage, myelomonocytic lineage or growth-arrested without differentiation by using appropriate agents. We have shown that H-, K-, and N-*ras* genes expression decreases during erythroid differentiation induced by arabinofuranosyl cytosine (ara-C) and myelomonocytic differentiation induced by the phorbol ester TPA and staurosporine.
However, in all the cytostatic treatments, the down-regulation of H-*ras* gene was much greater that the other two *ras* genes. The expression of H-*ras* at the mRNA and protein level became almost undetectable after three days of treatment with ara-C and TPA. H-*ras* downregulation was also detected when cells are treated with agents that induce a reversible growth arrest, as interferon-α and hydroxiurea. When these agents were removed from the media, the cells reassumed growth and H-*ras* expression increased concomitantly. However, H-*ras* expression remained low or undetectable when ara-C or TPA were washed from the media, a result consistent with the irreversile cytostasis caused by these agents.

We have obtained K562 sublines stably transfected with normal and oncogenic (codon 12 mutated) H-*ras* genes. Stable transfectants carrying multiple copies of normal H-*ras* gene overexpressed the H-*ras* mRNA and protein and did not show any change in growth rate or extent of differentiation with ara-C or TPA. However, H-*ras* expression was higher during the cytostatic treatments than in parental cells. We conclude that H-*ras* is a growth-regulated gene in K562 and that its downregulation is a consequence of growth arrest.

In contrast to cells overexpressing the normal gene, K562 cells carrying mutated H-*ras* (Val^{12}) constitutively expressed showed a reduced growth rate. These transfected lines, however, were able to differentiate in response to TPA or ara-C. The same result was observed in K562 cells transfected with glucocortocoid-inducible H-*ras* oncogene. In the presence of 1 μM dexamethasone the cells showed a reduced growth rate with respect to cultures in the absence of inducer. In contrast, K562 sublines transfected with mutated murine N-*ras* gene did not show significant differences in growth rate or extent of differentiation. We conclude that the expression of H-*ras* oncogenic mutation impairs cell growth in K562 leukemia cells.

An oncogenic form of human raf can specify terminal body pattern in Drosophila

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Terminal portions of the Drosophila body pattern are specified by an extracellular ligand generated at each end of the early syncytial embryo. This ligand triggers the localized transcription of two gap segmentation genes, tailless (tll) and huckebein (hkb) through a signal transduction cascade involving the receptor tyrosine kinase torso (tor) and homologues of ras, raf, and map kinase-kinase. In contrast to the ligand, these signal transducing components are expressed ubiquitously. Here, we show that constitutively active form of human raf1 protein can trigger tll and hkb transcription in Drosophila embryos and specify elements of the terminal body pattern. This result indicates a strong functional conservation between Drosophila and mammalian raf proteins and argues that the localized activity of Drosophila raf (D-raf) normally carries spatial information specifying the end portions of the body.

THE ROLE OF RAS IN MYELOID LEUKAEMOGENESIS - STUDIES USING RETROVIRAL-MEDIATED GENE TRANSFER.

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The role of mutant ras in leukaemogenesis is being studied using retrovirus-mediated gene transfer of mutant H-ras, N-ras and K-ras genes into a variety of haemopoietic cell types including normal human marrow stem cells, preleukaemic stem cells and preleukaemic and leukaemic cell lines.

Normal bone marrow cells infected with a mutant H-ras-containing retrovirus (codon 12 gly-> asp) were established in long-term culture. Compared to marrow cultures infected with a control retrovirus, the mutant H-ras-infected marrow cells proliferated to a greater extent and the proportion of primitive blast cells in the cultures increased. In addition, the number of mature myeloid cells fell. No leukaemic colonies were generated in the mutant H-ras-infected cultures and H12-ras did not confer serum independence. Together the data indicated that expression of mutant H12-ras in normal marrow progenitors favoured proliferation at the expense of differentiation but was insufficient to effect leukaemic transformation of normal cells. Similar experiments with mutant N-ras and K-ras (codon 12) retroviruses are currently in progress.

We have also used wild-type and mutant N-ras, H-ras and K-ras retroviruses to infect factor-dependent myeloid and lymphoid cell lines. While wild-type H-, N- and K-ras have no effect, their mutant counterparts allow the cells to proliferate to a variable degree in liquid culture and colony assays in the absence of IL-3 and GM-CSF. Experiments to evaluate the mechanism of these effects are ongoing.

In human acute myeloid leukaemia, ras mutations are the commonest identifiable genetic abnormalities. The only consistent differences between leukaemias with, as opposed to those without, ras mutations is the clear association between ras mutation and a monocytic phenotype. We have investigated this by infecting the monoblastic cell line, U937, with a mutant H-ras-containing retrovirus (PBH-H12-ras). A high proportion of the infected cells developed monocytic morphology (71% vs 11% infected with the control retrovirus) and immunological and functional characteristics of monocytes (phagocytosis, plastic adherence). There was no evidence that this was due to autocrine growth factor production. These findings suggest that ras plays a direct role in promoting monocytic differentiation in AML.

INHIBITION OF PROTEIN ISOPRENYLATION INDUCES APOPTOSIS IN HUMAN PROMYELOCYTIC HL-60 CELLS

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Protein isoprenylation is a posttranslational modification that facilitates membrane association and biological activity of a number of cellular proteins including p21Ras and nuclear lamins A and B. Mevalonate is the precursor of cellular sterols as well of isoprenoid lipids involved in protein modification.

In this study we show that HL-60 cells treated with lovastatin, an inhibitor of mevalonate synthesis, exhibit alterations in growth, morphology and function. Moreover, they are induced to die via apoptosis, which results in the appearance of a typical DNA fragmentation pattern. This process is in good correlation with lovastatin inhibition of protein isoprenylation, evidenced by the accumulation of p21Ras and nuclear lamin A in the soluble fraction of these cells.

Lovastatin induced DNA fragmentation can be specifically prevented by mevalonate. The failure of several products of the mevalonate pathway, including cholesterol, to overcome lovastatin effect, supports the involvement of isoprenylated proteins in the mechanisms suppressing cell death. The role of ras and ras-related proteins in these processes is under study.

CONCLUDING REMARKS

J. Downward

The past couple of years have seen an enormously rapid expansion of our knowledge of the regulation and function of Ras proteins. In May 1993 a spate of reports in the leading journals established a model for the regulation of the activation state of Ras in response to mitogens such as epidermal growth factor (EGF). The picture that emerged was this: stimulation of the EGF receptor by ligand binding leads to its autophosphorylation at several sites near its carboxy terminus. These bind to the SH2 domain of the Grb2 adaptor protein which in turn binds through its SH3 domains to the Ras-specific guanine nucleotide exchange factor Sos. This results in stimulation of the nucleotide exchange rate of Ras, either by translocating Sos from the cytosol to the plasma membrane where Ras is located, or by allosteric regulation, or perhaps a combination of the two. The increased exchange rate causes a larger proportion of Ras to enter the biologically active GTP-bound conformation. While the exact details of this mechanism are still to be defined and the degree to which other mitogens use the same pathway is not totally clear, knowledge of the upstream regulation of Ras has been revolutionised.

Similarly, knowledge of the downstream targets of Ras through which it exerts its mitogenic effects on cells was radically advanced in July 1993. At that time another rush of reports were published showing by a variety of approaches that GTP-bound Ras interacts directly with the serine/threonine

protein kinases of the Raf family through its effector site, the region on Ras mutationally defined to be crucial for its transforming ability. Again, this is certainly not the end of the story: it is still not known how, or whether, this interaction controls the kinase activity of Raf and it is certainly possible, indeed probable, that there exist other effectors of Ras in addition to Raf. However, once more a revolution has occurred, this time in understanding pathways downstream of Ras.

Most of these advances have occurred since the original planning of this meeting. Despite the enormous progress that has already been made, it was therefore most rewarding to see how much is still being achieved in a wide variety of areas. On the basic mechanisms of Ras function and regulation, progress was reported on the characterisation of guanine nucleotide exchange factors for Ras, particularly CDC25 in yeast (A.Levitzki), Ras-GRF in mammalian brain (D.Lowy, L.Feig), although the regulatory mechanisms for these factors remain less well characterised than for Sos. A novel inhibitor of guanine nucleotide release from Ras, or GDI, was reported by G.Bollag. This protein is identical to that encoded by the *Set* gene, a very abundant cellular protein; interestingly, Set has the opposite effect on K-Ras as it does on N-Ras. Downstream of Ras, C.Marshall provided compelling evidence that the most important aspect of the interaction of Ras and Raf is to bring Raf to the plasma membrane where its kinase activity is turned on by some other, as yet unknown, component. He also presented data showing that MAP kinase kinase is the key target of Ras action in the regulation of cell proliferation in NIH 3T3 cells and differentiation in PC12 cells. The picture emerging is that the MAP kinase pathway is entirely responsible for the main growth stimulatory signal emerging from Ras, although other effects of Ras, such as morphological changes, could be due to other effectors. Ras has been reported to interact with a number of potential targets other than Raf, including $p120^{GAP}$, neurofibromin and Ral-GDS. In addition, I reported work from P.Rodriguez in

my own group indicating that Ras can interact with the regulatory subunit of phosphatidyl inositol 3' kinase, possibly directly, and can cause elevation of phosphatidyl inositol (3,4,5) trisphosphate levels in intact cells. Thus phosphatidyl inositol 3' kinase could represent an alternative effector pathway for Ras, possibly mediating effects on cell morphology.

The emphasis in this meeting was on the function of Ras in differentiation and development, rather than simply in the control of cell growth. Two model systems in which activation of Ras leads to differentiation were discussed: the PC12 cell line, in which nerve growth factor acts through Ras to induce differentiation to a sympathetic neuron-like cell (C.Marshall), and 3T3-L1 cells, in which insulin acting through Ras leads to differentiation to adipocytes (E.Santos). In the case of PC12 cells, both epidermal growth factor (EGF) and nerve growth factor (NGF) cause activation of Ras, but only NGF leads to differentiation. The difference between the two factors appears to be principally the duration of the activation of Ras and MAP kinase. Overexpression of the EGF receptor in PC12 cells gives a prolonged activation of Ras and MAP kinase and allows EGF to induce differentiation rather than cell growth (C.Marshall).

Within the whole mammalian organism, the importance of Ras in the generation of cancer has long been recognised. Transgenic mouse work has added to this knowledge (A.Balmain), while the creation of mice lacking N-Ras has shown that it is not required for development (A.Pellicer). Presumably this reflects the redundancy of the Ras family, with three members, H-, K- and N-Ras, all of which appear to be functionally interchangeable. It will be very interesting to learn the phenotype of mice lacking other Ras genes, and of the effects of interbreeding them.

Two other organisms of great importance in research on Ras were discussed: the nematode worm, *C. elegans* (K.Kornfeld), and the fruit fly, *D.melanogaster* (F.Karim). The involvement of Ras in the vulval development

pathway in the worm and the development of photoreceptor R7 in the fly has provided enormous amounts of genetic information about the components upstream and downstream of Ras, most notably the exchange factor Sos in flies and the adaptor Sem-5 in worms. Work in these organisms is now largely focusing on the transcription factors downstream of Ras: these include genes encoding Ets-like proteins, such as *yan* and *pointed* in flies, and *lin-1* in worms. In addition, a number of negative regulators of vulval induction have been characterised and found to be novel proteins (lin-9, lin-15a, lin-15b and lin-36).

The meeting provided a stimulating environment for researchers working on diverse aspects of Ras to come together in an intimate environment. Clearly, although great progress has been made and continues to be reported, there is still a long way to go before the functions of Ras proteins are fully understood, especially with regard to development and differentiation. Many key issues remain to be resolved, such as the level of redundancy of function of the Ras proteins and the complexity of the signalling pathways in which they are involved. These issues may be resolved within the next couple of years, or at least by the time the Juan March Foundation has another workshop on this subject.

List of Invited Speakers

Workshop on

RAS, DIFFERENTIATION AND DEVELOPMENT

List of Invited Speakers

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Workshop on

RAS, DIFFERENTIATION AND DEVELOPMENT

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Organized by P. Marrack and C. Martínez-A. Lectures by H. von Boehmer, J. W. Kappler, C. Martínez-A., H. Waldmann, N. Le Douarin, J. Sprent, P. Matzinger, R. H. Schwartz, M. Weigert, A. Coutinho, C. C. Goodnow, A. L. DeFranco and P. Marrack.

247 **Workshop on Pathogenesis-related Proteins in Plants.**
Organized by V. Conejero and L. C. Van Loon. Lectures by L. C. Van Loon, R. Fraser, J. F. Antoniw, M. Legrand, Y. Ohashi, F. Meins, T. Boller, V. Conejero, C. A. Ryan, D. F. Klessig, J. F. Bol, A. Leyva and F. García-Olmedo.

248 Beato, M.:
Course on DNA - Protein Interaction.

249 **Workshop on Molecular Diagnosis of Cancer.**
Organized by M. Perucho and P. García Barreno. Lectures by F. McCormick, A. Pellicer, J. L. Bos, M. Perucho, R. A. Weinberg, E. Harlow, E. R. Fearon, M. Schwab, F. W. Alt, R. Dalla Favera, P. E. Reddy, E. M. de Villiers, D. Slamon, I. B. Roninson, J. Groffen and M. Barbacid.

251 **Lecture Course on Approaches to Plant Development.**
Organized by P. Puigdoménech and T. Nelson. Lectures by I. Sussex, R. S. Poethig, M. Delseny, M. Freeling, S. C. de Vries, J. H. Rothman, J. Modolell, F. Salamini, M. A. Estelle, J. M. Martínez Zapater, A. Spena, P. J. J. Hooykaas, T. Nelson, P. Puigdoménech and M. Pagès.

252 **Curso Experimental de Electroforesis Bidimensional de Alta Resolución.**
Organizado por Juan F. Santarén. Seminarios por Julio E. Celis, James I. Garrels, Joël Vandekerckhove, Juan F. Santarén y Rosa Assiego.

253 **Workshop on Genome Expression and Pathogenesis of Plant RNA Viruses.**
Organized by F. García-Arenal and P. Palukaitis. Lectures by D. Baulcome, R. N. Beachy, G. Boccardo, J. Bol, G. Bruening, J. Burgyan, J. R. Díaz Ruiz, W. G. Dougherty, F. García-Arenal, W. L. Gerlach, A. L. Haenni, E. M. J. Jaspars, D. L. Nuss, P. Palukaitis, Y. Watanabe and M. Zaitlin.

254 **Advanced Course on Biochemistry and Genetics of Yeast.**
Organized by C. Gancedo, J. M. Gancedo, M. A. Delgado and I. L Calderón.

255 **Workshop on The Reference Points in Evolution.**
Organized by P. Alberch and G. A. Dover. Lectures by P. Alberch, P. Bateson, R. J. Britten, B. C. Clarke, S. Conway Morris, G. A. Dover, G. M. Edelman, R. Flavell, A. Fontdevila, A. García-Bellido, G. L. G. Miklos, C. Milstein, A. Moya, G. B. Müller, G. Oster, M. De Renzi, A. Seilacher, S. Stearns, E. S. Vrba, G. P Wagner, D. B. Wake and A. Wilson.

256 **Workshop on Chromatin Structure and Gene Expression.**
Organized by F. Azorín, M. Beato and A. A. Travers. Lectures by F. Azorín, M. Beato, H. Cedar, R. Chalkley, M. E. A. Churchill, D. Clark, C. Crane-Robinson, J. A. Dabán, S. C. R. Elgin, M. Grunstein, G. L. Hager, W. Hörz, T. Koller, U. K. Laemmli, E. Di Mauro, D. Rhodes, T. J. Richmond, A. Ruiz-Carrillo, R. T. Simpson, A. E. Sippel, J. M. Sogo, F. Thoma, A. A. Travers, J. Workman, O. Wrange and C. Wu.

257 **Lecture Course on Polyamines as modulators of Plant Development.**
Organized by A. W. Galston and A. F. Tiburcio. Lectures by N. Bagni, J. A. Creus, E. B. Dumbroff, H. E. Flores, A. W. Galston, J. Martin-Tanguy, D. Serafini-Fracassini, R. D. Slocum, T. A. Smith and A. F. Tiburcio.

258 **Workshop on Flower Development.**
Organized by H. Saedler, J. P. Beltrán and J. Paz Ares. Lectures by P. Albersheim, J. P. Beltrán, E. Coen, G. W. Haughn, J. Leemans, E. Lifschitz, C. Martin, J. M. Martínez-Zapater, E. M. Meyerowitz, J. Paz-Ares, H. Saedler, C. P. Scutt, H. Sommer, R. D. Thompson and K. Tran Thahn Van.

259 **Workshop on Transcription and Replication of Negative Strand RNA Viruses.**
Organized by D. Kolakofsky and J. Ortín. Lectures by A. K. Banerjee, M. A. Billeter, P. Collins, M. T. Franze-Fernández, A. J. Hay, A. Ishihama, D. Kolakofsky, R. M. Krug, J. A. Melero, S. A. Moyer, J. Ortín, P. Palese, R. G. Paterson, A. Portela, M. Schubert, D. F. Summers, N. Tordo and G. W. Wertz.

260 **Lecture Course Molecular Biology of the Rhizobium-Legume Symbiosis.**
Organized by T. Ruiz-Argüeso. Lectures by T. Bisseling, P. Boistard, J. A. Downie, D. W. Emerich, J. Kijne, J. Olivares, T. Ruiz-Argüeso, F. Sánchez and H. P. Spaink.

261 **Workshop The Regulation of Translation in Animal Virus-Infected Cells.**
Organized by N. Sonenberg and L. Carrasco. Lectures by V. Agol, R. Bablanian, L. Carrasco , M. J. Clemens, E. Ehrenfeld, D. Etchison, R. F. Garry, J. W. B. Hershey, A. G. Hovanessian, R. J. Jackson, M. G. Katze, M. B. Mathews, W. C. Merrick, D. J. Rowlands, P. Sarnow, R. J. Schneider, A. J. Shatkin, N. Sonenberg, H. O. Voorma and E. Wimmer.

263 **Lecture Course on the Polymerase Chain Reaction.**
Organized by M. Perucho and E. Martínez-Salas. Lectures by D. Gelfand, K. Hayashi, H. H. Kazazian, E. Martínez-Salas, M. Mc Clelland, K. B. Mullis, C. Oste, M. Perucho and J. Sninsky.

264 **Workshop on Yeast Transport and Energetics.**
Organized by A. Rodríguez-Navarro and R. Lagunas. Lectures by M. R. Chevallier, A. A. Eddy, Y. Eilam, G. F. Fuhrmann, A. Goffeau, M. Höfer, A. Kotyk, D. Kuschmitz, R. Lagunas, C. Leão, L. A. Okorokov, A. Peña, J. Ramos, A. Rodríguez-Navarro, W. A. Scheffers and J. M. Thevelein

265 **Workshop on Adhesion Receptors in the Immune System.**
Organized by T. A. Springer and F. Sánchez-Madrid. Lectures by S. J. Burakoff, A. L. Corbi-López, C. Figdor, B. Furie, J. C. Gutiérrez-Ramos, A. Hamann, N. Hogg, L. Lasky, R. R. Lobb, J. A. López de Castro, B. Malissen, P. Moingeon, K. Okumura, J. C. Paulson, F. Sánchez-Madrid, S. Shaw, T. A. Springer, T. F. Tedder and A. F. Williams.

266 **Workshop on Innovations on Proteases and their Inhibitors: Fundamental and Applied Aspects.**
Organized by F. X. Avilés. Lectures by T. L. Blundell, W. Bode, P. Carbonero, R. W.Carrell, C. S. Craik, T. E. Creighton, E. W. Davie, L. D. Fricker, H. Fritz, R. Huber, J. Kenny, H. Neurath, A. Puigserver, C. A. Ryan, J. J. Sánchez-Serrano, S. Shaltiel, R. L. Stevens, K. Suzuki, V. Turk, J. Vendrell and K. Wüthrich.

267 **Workshop on Role of Glycosyl-Phosphatidylinositol in Cell Signalling.**
Organized by J. M. Mato and J. Larner. Lectures by M. V. Chao, R. V. Farese, J. E. Felíu, G. N. Gaulton, H. U. Häring, C. Jacquemin, J. Larner, M. G. Low, M. Martín Lomas, J. M. Mato, E. Rodríguez-Boulan, G. Romero, G. Rougon, A. R. Saltiel, P. Strålfors and I. Varela-Nieto.

268 **Workshop on Salt Tolerance in Microorganisms and Plants: Physiological and Molecular Aspects.**
Organized by R. Serrano and J. A. Pintor-

Toro. Lectures by L. Adler, E. Blumwald, V. Conejero, W. Epstein, R. F. Gaber, P. M. Hasegawa, C. F. Higgins, C. J. Lamb, A. Läuchli, U. Lüttge, E. Padan, M. Pagès, U. Pick, J. A. Pintor-Toro, R. S. Quatrano, L. Reinhold, A. Rodríguez-Navarro, R. Serrano and R. G. Wyn Jones.

269 **Workshop on Neural Control of Movement in Vertebrates.**
Organized by R. Baker and J. M. Delgado-García. Lectures by C. Acuña, R. Baker, A. H. Bass, A. Berthoz, A. L. Bianchi, J. R. Bloedel, W. Buño, R. E. Burke, R. Caminiti, G. Cheron, J. M. Delgado-García, E. E. Fetz, R. Gallego, S. Grillner, D. Guitton, S. M. Highstein, F. Mora, F. J. Rubia Vila, Y. Shinoda, M. Steriade and P. L. Strick.

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Organized by C. Belmonte and F. Cerveró. Lectures by C. Belmonte, G. J. Bennet, J. N. Campbell, F. Cerveró, A. W. Duggan, J. Gallar, H. O. Handwerker, M. Koltzenburg, R. H. LaMotte, R. A. Meyer, J. Ochoa, E. R. Perl, H. P. Rang, P. W. Reeh, H. G. Schaible, R. F. Schmidt, J. Szolcsányi, E. Torebjörk and W. D. Willis Jr.

2 **Workshop on DNA Structure and Protein Recognition.**
Organized by A. Klug and J. A. Subirana. Lectures by F. Azorín, D. M. Crothers, R. E. Dickerson, M. D. Frank-Kamenetskii, C. W. Hilbers, R. Kaptein, D. Moras, D. Rhodes, W. Saenger, M. Salas, P. B. Sigler, L. Kohlstaedt, J. A. Subirana, D. Suck, A. Travers and J. C. Wang.

3 **Lecture Course on Palaeobiology: Preparing for the Twenty-First Century.**
Organized by F. Álvarez and S. Conway Morris. Lectures by F. Álvarez, S. Conway Morris, B. Runnegar, A. Seilacher and R. A. Spicer.

4 **Workshop on The Past and the Future of Zea Mays.**
Organized by B. Burr, L. Herrera-Estrella and P. Puigdoménech. Lectures by P. Arruda, J. L. Bennetzen, S. P. Briggs, B. Burr, J. Doebley, H. K. Dooner, M. Fromm, G. Gavazzi, C. Gigot, S. Hake, L. Herrera-Estrella, D. A. Hoisington, J. Kermicle, M. Motto, T. Nelson, G. Neuhaus, P. Puigdomenech, H. Saedler, V. Szabo and A. Viotti.

5 **Workshop on Structure of the Major Histocompatibility complex.**
Organized by A. Arnaiz-Villena and P. Parham. Lectures by A. Arnaiz-Villena, R. E. Bontrop, F. M. Brodsky, R. D. Campbell, E. J. Collins, P. Cresswell, M. Edidin, H. Erlich, L. Flaherty, F. Garrido, R. Germain, T. H. Hansen, G. J. Hämmerling, J. Klein, J. A. López de Castro, A. McMichael, P. Parham, P. Stastny, P. Travers and J. Trowsdale.

6 **Workshop on Behavioural Mechanisms in Evolutionary Perspective.**
Organized by P. Bateson and M. Gomendio. Lectures by J. R. Alberts, G. W. Barlow, P. Bateson, T. R. Birkhead, J. Carranza, C. ten Cate, F. Colmenares, N. B. Davies, R. I. M. Dunbar, J. A. Endler, M. Gomendio, T. Guilford, F. Huntingford, A. Kacelnik, J. Krebs, J. Maynard Smith, A. P. Møller, J. Moreno, G. A. Parker, T. Redondo, D. I. Rubenstein, M. J. Ryan, F. Trillmich and J. C. Wingfield.

7 **Workshop on Transcription Initiation in Prokaryotes.**
Organized by M. Salas and L. B. Rothman-Denes. Lectures by S. Adhya, H. Bujard, S. Busby, M. J. Chamberlin, R. H. Ebright, M. Espinosa, E. P. Geiduschek, R. L. Gourse, C. A. Gross, S. Kustu, J. Roberts, L. B. Rothman-Denes, M. Salas, R. Schleif, P. Stragier and W. C. Suh.

8 **Workshop on the Diversity of the Immunoglobulin Superfamily.**
Organized by A. N. Barclay and J. Vives. Lectures by A. N. Barclay, H. G. Boman, I. D. Campbell, C. Chothia, F. Díaz de Espada, I. Faye, L. García Alonso, P. R. Kolatkar, B. Malissen, C. Milstein, R. Paolini, P. Parham, R. J. Poljak, J. V. Ravetch, J. Salzer, N. E. Simister, J. Trinick, J. Vives, B. Westermark and W. Zimmermann.

9 **Workshop on Control of Gene Expression in Yeast.**
Organized by C. Gancedo and J. M. Gancedo. Lectures by T. G. Cooper, T. F. Donahue, K.-D. Entian, J. M. Gancedo, C. P. Hollenberg, S. Holmberg, W. Hörz, M. Johnston, J. Mellor, F. Messenguy, F. Moreno, B. Piña, A. Sentenac, K. Struhl, G. Thireos and R. S. Zitomer.

10 **Workshop on Engineering Plants Against Pests and Pathogens.**
Organized by G. Bruening, F. García-Olmedo and F. Ponz. Lectures by R. N. Beachy, J. F. Bol, T. Boller, G. Bruening, P. Carbonero, J. Dangl, R. de Feyter, F. García-Olmedo, L. Herrera-Estrella, V. A. Hilder, J. M. Jaynes, F. Meins, F. Ponz, J. Ryals, J. Schell, J. van Rie, P. Zabel and M. Zaitlin.

11 **Lecture Course on Conservation and Use of Genetic Resources.**
Organized by N. Jouve and M. Pérez de la Vega. Lectures by R. P. Adams, R. W. Allard, T. Benítez, J. I. Cubero, J. T. Esquinas-Alcázar, G. Fedak, B. V. Ford-Lloyd, C. Gómez-Campo, V. H. Heywood, T. Hodgkin, L. Navarro, F. Orozco, M. Pérez de la Vega, C. O. Qualset, J. W. Snape and D. Zohary.

12 **Workshop on Reverse Genetics of Negative Stranded RNA Viruses.**
Organized by G. W. Wertz and J. A. Melero. Lectures by G. M. Air, L. A. Ball, G. G. Brownlee, R. Cattaneo, P. Collins, R. W. Compans, R. M. Elliott, H.-D. Klenk, D. Kolakofsky, J. A. Melero, J. Ortín, P. Palese, R. F. Pettersson, A. Portela, C. R. Pringle, J. K. Rose and G. W. Wertz.

13 **Workshop on Approaches to Plant Hormone Action.**
Organized by J. Carbonell and R. L. Jones. Lectures by J. P. Beltrán, A. B. Bleecker, J. Carbonell, R. Fischer, D. Grierson, T. Guilfoyle, A. Jones, R. L. Jones, M. Koornneef, J. Mundy, M. Pagès, R. S. Quatrano, J. I. Schroeder, A. Spena, D. Van Der Straeten and M. A. Venis.

14 **Workshop on Frontiers of Alzheimer Disease.**
Organized by B. Frangione and J. Ávila. Lectures by J. Ávila, K. Beyreuther, D. D. Cunningham, A. Delacourte, B. Frangione, C. Gajdusek, M. Goedert, Y. Ihara, K. Iqbal, K. S. Kosik, C. Milstein, D. L. Price, A. Probst, N. K. Robakis, A. D. Roses, S. S. Sisodia, C. J. Smith, W. G. Turnell and H. M. Wisniewski.

15 **Workshop on Signal Transduction by Growth Factor Receptors with Tyrosine Kinase Activity.**
Organized by J. M. Mato and A. Ullrich. Lectures by M. Barbacid, A. Bernstein, J. B. Bolen, J. Cooper, J. E. Dixon, H. U. Häring, C.- H. Heldin, H. R. Horvitz, T. Hunter, J. Martín-Pérez, D. Martín-Zanca, J. M. Mato, T. Pawson, A. Rodríguez-Tébar, J. Schlessinger, S. I. Taylor, A. Ullrich, M. D. Waterfield and M. F. White.

16 **Workshop on Intra- and Extra-Cellular Signalling in Hematopoiesis.**
Organized by E. Donnall Thomas and A. Grañena. Lectures by M. A. Brach, D. Cantrell, L. Coulombel, E. Donnall Thomas, M. Hernández-Bronchud, T. Hirano, L. H. Hoefsloot, N. N. Iscove, P. M. Lansdorp, J. J. Nemunaitis, N. A. Nicola, R. J. O'Reilly, D. Orlic, L. S. Park, R. M. Perlmutter, P. J. Quesenberry, R. D. Schreiber, J. W. Singer and B. Torok-Storb.

17 **Workshop on Cell Recognition During Neuronal Development.**
Organized by C. S. Goodman and F. Jiménez. Lectures by J. Bolz, P. Bovolenta, H. Fujisawa, C. S. Goodman, M. E. Hatten, E. Hedgecock, F. Jiménez, H. Keshishian, J. Y. Kuwada, L. Landmesser, D. D. M. O'Leary, D. Pulido, J. Raper, L. F. Reichardt, M. Schachner, M. E. Schwab, C. J. Shatz, M. Tessier-Lavigne, F. S. Walsh and J. Walter.

18 **Workshop on Molecular Mechanisms of Macrophage Activation.**
Organized by C. Nathan and A. Celada. Lectures by D. O. Adams, M. Aguet, C. Bogdan, A. Celada, J. R. David, A. Ding, R. M. Fauve, D. Gemsa, S. Gordon, J. A. M. Langermans, B. Mach, R. Maki, J. Mauël, C. Nathan, M. Röllinghoff, F. Sánchez-Madrid, C. Schindler, A. Sher and Q.-w. Xie.

19 Workshop on Viral Evasion of Host Defense Mechanisms.
Organized by M. B. Mathews and M. Esteban. Lectures by E. Domingo, L. Enjuanes, M. Esteban, B. N. Fields, B. Fleckenstein, L. Gooding, M. S. Horwitz, A. G. Hovanes-sian, M. G. Katze, I. M. Kerr, E. Kieff, M. B. Mathews, G. McFadden, B. Moss, J. Pavlovic. P. M. Pitha, J. F. Rodríguez, R. P. Ricciardi, R. H. Silverman, J. J. Skehel, G. L. Smith, P. Staeheli, A. J. van der Eb, S. Wain-Hobson, B. R. G. Williams and W. Wold.

20 Workshop on Genomic Fingerprinting.
Organized by McClelland and X. Estivill. Lectures by G. Baranton, D. E. Berg, X. Estivill, J. L. Guénet, K. Hayashi, H. S. Kwan, P. Liang, M. McClelland, J. H. Nadeau, D. L. Nelson, M. Perucho, W. Powell, J. A. Rafalski, O. A. Ryder, R. Sederoff, A. J. G. Simpson, J. Welsh and H. Zischler.

21 Workshop on DNA-Drug Interactions.
Organized by K. R. Fox and J. Portugal. Lectures by J. B. Chaires, W. A. Denny, R. E. Dickerson, K. R. Fox, F. Gago, T. Garestier, I. H. Goldberg, T. R. Krugh, J. W. Lown, L. A. Marky, S. Neidle, D. J. Patel, J. Portugal, A. Rich, L. P. G. Wakelin, M. J. Waring and C. Zimmer.

22 Workshop on Molecular Bases of Ion Channel Function.
Organized by R. W. Aldrich and J. López-Barneo. Lectures by R. W. Aldrich, C. M. Armstrong, P. Ascher, K. G. Beam, F. Bezanilla, S. C. Cannon, A. Castellano, D. Clapham, A. Ferrús, T. Hoshi, A. Konnerth, R. Latorre, J. Lerma, J. López-Barneo, R. MacKinnon, G. Mandel, A. Marty, C. Miller, B. Sakmann, B. Soria, W. Stühmer and W. N. Zagotta.

23 Workshop on Molecular Biology and Ecology of Gene Transfer and Propagation Promoted by Plasmids.
Organized by C. M. Thomas, E. M. H. Wellington, M. Espinosa and R. Díaz Orejas. Lectures by J. C. Alonso, F. Baquero, P. A. Battaglia, P. Courvalin, F. de la Cruz, D. K. Chattoraj, E. Díaz, R. Díaz Orejas, M. Espinosa, J. C. Fry, K. Gerdes, D. R. Helinski, B. Hohn, E. Lanka, V. de Lorenzo, S. Molin, K. Nordström, R. W. Pickup, C. M. Thomas, J. D. van Elsas, E. M. H. Wellington and B. Wilkins.

24 Workshop on Deterioration, Stability and Regeneration of the Brain During Normal Aging.
Organized by P. D. Coleman, F. Mora and M. Nieto-Sampedro. Lectures by J. Avila, Y.-A. Barde, P. D. Coleman, C. W. Cotman, Y. Lamour, M. P. Mattson, A. Matus, E. G. McGeer, B. S. Meldrum, J. Miquel, F. Mora, M. Nieto-Sampedro, V. H. Perry, M. P. Rathbone, G. S. Roth, R. R. Sturrock, H. B. M. Uylings and M. J. West.

25 Workshop on Genetic Recombination and Defective Interfering Particles in RNA Viruses.
Organized by J. J. Bujarski, S. Schlesinger and J. Romero. Lectures by V. Agol, P. Ahlquist, J. J. Bujarski, J. Burgyán, E. Domingo, L. Enjuanes, S. P. Goff, T. C. Hall, A. S. Huang, K. Kirkegaard, M. M. C. Lai, T. J. Morris, R. F. Ramig, D. J. Robinson, J. Romero, L. Roux, S. Schlesinger, A. E. Simon, W. J. M. Spaan, E. G. Strauss and S. Wain-Hobson.

26 Workshop on Cellular Interactions in the Early Development of the Nervous System of Drosophila.
Organized by J. Modolell and P. Simpson. Lectures by S. Artavanis-Tsakonas, J. A. Campos-Ortega, S. B. Carroll, C. Dambly-Chaudière, C. Q. Doe, R. G. Fehon, M. Freeman, A. Ghysen, V. Hartenstein, D. Hartley, Y. N. Jan, C. Klämbt, E. Knust, A. Martínez-Arias, M. Mlodzik, J. Modolell, M. A. T. Muskavitch, B.- Z. Shilo, P. Simpson, G. M. Technau, D. van Vactor and C. Zuker.

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