Instituto Juan March
de Estudios e Investigaciones

48 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

G-Proteins: Structural Features and Their Involvement in the Regulation of Cell Growth

Organized by

B. F. C. Clark and J. C. Lacal

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INTRODUCTION AND SUMMARY

Brian F. C. Clark and
Juan Carlos Lacal

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G PROTEINS: STRUCTURAL FEATURES AND THEIR INVOLVEMENT IN THE REGULATION OF CELL GROWTH

G-proteins are defined from their ability to bind guanine nucleotides, guanosine diphospate (GDP) and guanosine triphosphate (GTP). When bound to either GDP or GTP, they acquire distinct tertiary conformations that can enable the complex to interact specifically with key enzymes involved in the regulation of essential metabolic processes. These proteins perform a wide variety of biological functions being involved with protein biosynthesis, cell growth, cell differentiation and apoptosis, signal transduction, membrane traffic and secretion and cell skeletal organisation and movement.

There are at least three distinct super families of G proteins. Each super family has multiple members grouped in gene families that have a great degree of conservation along evolution. Proteins involved in the regulation of protein synthesis, which best representative is EF-Tu, belongs to the first group of classical G-proteins. A second group includes the heterotrimeric G proteins, which are made of three different subunits ($\alpha\beta\gamma$). Only the α subunit has the ability to bind and hydrolyse GTP, and exert their function when associated to GTP. On the other hand, the $\beta\gamma$ subunits play regulatory functions for the α subunits, but can also regulate some effector-mediated functions themselves. Classical examples of this class of G proteins are those involved in the regulation of the adenylate cyclase, transducin or PI-specific phospholipase C β (PI-PLC β). The third group is composed of monomeric G proteins, which are characterised for the existence of only one subunit, but they are also regulated in a similar way as the heterotrimeric proteins by other components. The most relevant members of this superfamily are the Ras oncoproteins. Tubulin, a protein that has the ability to bind and hydrolyse GTP, makes a fourth category of G-proteins with a function in microtubules organisation distinct from the other three groups.

In the last few years, it has been a dramatic progress in the understanding on the function of G proteins. This is due to the deciphering of the tertiary structure of some of the members of each family. Comparison of these structures has been useful to locate the specific regions of each

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molecule involved in the interaction with the effectors and regulatory proteins.

Progress in recent years in the understanding of the mechanisms of regulation of enzymatic activities where G proteins are involved, has made clear the relevance of these molecules in the regulation of many cellular processes. Among these, regulation of protein synthesis, signal transduction in cell growth, differentiation, and apoptosis, membrane traffic and secretion, and cell skeletal organisation and movement, are some of the most important, due to their implications in biomedical research.

A better understanding of the mechanisms of regulation of the G proteins involved in theses biological processes, may help in the design of strategies to fight human diseases. On this regard, it is important to note the discovery of the presence of mutated genes for G proteins, either monomeric or heterotrimeric, in numerous types of human tumours, while alterations of the regulatory components of some GTPases have been related to human disorders which prone to malignancy (neurofibromatosis, tuberous sclerosis complex, myotonic dystrophy, Wiscott-Aldrich syndrome, etc.). Direct involvement of GTPases is also known in normal essential functions such as sense transduction systems (vision, taste, smell), and diseases other than cancer such as diabetes. Thus, the study of the structure/function relationship of G proteins is one of the most exciting fields of research nowadays, and has a direct application to human health.

SUMMARY OF THE MEETING

The aim of the workshop was to review state-of-the-art information available on G-protein three-dimensional structures and to try to see how they explain biological phenomena caused by G-proteins. Common to the functioning of G-proteins is activation of the G-protein which occurs by binding GTP whereas deactivation is achieved by hydrolysis to GDP and inorganic phosphate. The substitution of GTP for GDP of the G-protein is usually catalysed by a guanine nucleotide exchange factor (GEF). Stimulation of the GTPase activity is controlled by a GTPase activator protein (GAP). Three or four consensus sequence elements have been detected in all G-proteins except in those concerned with movement.

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It was reported at the workshop that tubulins which bind GTP do not contain the common sequence motifs of other G-proteins and the spatial structure is still unknown. Three-dimensional G-protein structures are available and were discussed for the GDP and GTP forms of the protein translation factor, elongation factor Tu (the first G-protein structure to be solved), ras p21 protein, Ran protein, transducin $G_{t\alpha}$, the inhibitory G-protein subunit, $G_{i\alpha 1}$, and the G-protein ADP ribosylation factor (ARF) involved in secretion. The recently published complex of a Ras-type protein rap1A-GTP analogue complex with a piece of its effector molecular C-Raf 1 was also discussed to shed light on details of the p21 Ras involvement in signal transduction and the oncogenesis pathway.

Exciting unpublished structural information was reported for other complexes of G-proteins at the workshop. The ternary complex of aminoacyl-tRNA, elongation factor Tu and a GTP analogue has provided further information on how a coeffector binds to a G-protein for transmitting information. Surprisingly this structure is very similar to a recently reported structure of translation elongation factor EF-G suggesting a common ribosomal binding site. The tRNA part and three EF-G-domains are so similar that the concept of macromolecular mimicry has arisen. This idea proposes that structural and functional mimicry of proteins and RNA should occur in other areas of biological activity. In addition details of a complex of EF-Tu:GTP and an analogue of aminoacyl adenine were reported confirming the structure of the binding pocket of the amino-acid end of aminoacyl-tRNA on the EF-Tu.

Further fascinating new structural information was given at the workshop about the spatial structure of a complete heteromer G-protein not just the α-subunit as previously reported. Since the $\beta\gamma$-subunit complex has functional activity of its own the $G_{\alpha\beta\gamma}$-structure will shed light on the molecular details of this important step in signal transduction.

Interesting biochemical and protein engineering analysis to relate structure and function were discussed in relation to the heat-stable elongation factor EF-Tu and a protein. α-gustducin involved in interaction with bitter taste receptors. New data were also presented on interaction of retinal cyclic GMP phosphorodiesterase inhibitor with transducin and on an adaptor molecule concerned with activation of the Ras pathway.

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The more structurally oriented part of the workshop was rounded off with a stimulating review of the mechanism of GTP-hydrolysis, which is still not solved in a general fashion by the known structures. The possible mechanism via a metaphosphate intermediate was a novel suggestion in this respect.

Regarding the function of G-proteins, there were reports on further details on the signalling routes affected by different members of the Ras superfamily. These pathways are now more complex that expected and both small and heterotrimeric G-proteins use same intracellular kinases cascades to trigger distinct cell responses. Thus, mitogenic activity of thrombin is blocked by neutralising antibodies to the heterotrimeric $G\alpha_{12}$, while the M1 muscarinic acetylcholine receptors use the $\beta\gamma$ subunits to activate the Ras/Raf/MAPK pathway. The participation of the RAF/MAPK pathway along with the SAPK/JNK pathway to selectively turn on specific signals was demonstrated for thrombin and anysomicine (stress) treatment by means of elegant recombination experiments where quimeric constructs between $p44^{MAPK}$ and $p38^{JNK}$ with altered specificity were obtained.

Of interest were several reports relating human diseases with some of the components of the regulatory mechanisms of G-proteins. Among these, tuberin, the product of the TSC2 gene involved in Tuberous sclerosis, functions as a GAP for Rap1; ROCK, a new effector for RhoA, has homology to the MD-PK (Myotonic dystrophy); Wasp, the product of the gene involved in the Wiskott-Aldrich syndrome specifically binds to CDC42; and Rad, a ras-related protein associated to diabetes, interacts with β-tropomyosin. Generation of mice deficient for P120-Ras-GAP or NF1 (the neurofibromatosis gene product), two GTPase activating molecules for the Ras proteins, indicated that while NF1 seems to be related to the basal regulation of the amount of Ras-GTP, GAP is responsible for the inactivation of Ras after transient induction by growth factors. Neither one of these two molecules were essential for MAPK activation and DNA synthesis induced by PDGF.

Of great relevance were also the reports of the increasing evidence demonstrating the implication of Rho proteins (including CDC42 and Rac) in signal transduction pathways. Thus, it was reported that these proteins regulate cell growth (Rho and Rac are oncogenes when activated by single amino acid substitutions), response to stress (CDC42 and Rac1 activate

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SAPK/JNK) transcriptional activation by SRF (RhoA, CDC42 and Rac1), and apoptosis (Rho proteins induce apoptosis after serum deprivation). In yeast, Rho has been related to PKC that activates the MAPK cascade for the maintenance of the cell wall integrity, and with other unknown effectors for bud formation. Furthermore, it was reported that Rho proteins can regulate cell growth and apoptosis depending upon the specific activation of endogenous enzymes such as PC-PLD and sphingomyelinase. Also a novel signalling pathway was demonstrated for Rho proteins (RhoA, RhoB, CDC42 and Rac1) since they all activate the NF κB transcription factor. Finally, evidence was provided that Rho proteins (both human Rho A and B and Rac 1) cooperate with Ras for full transformation and that they are required for the transforming activity of Ras proteins.

Profuse usage of the yeast two-hybrid system demonstrated the existence of new effector molecules for Rho proteins as well as Rap proteins. Among the former, at least PKC and other related kinases such as PKN, Rhophilin, Rock, Rhotekin and Citron were shown to directly interact with GTP-bound Rho proteins. Furthermore, there seems to exist different effectors for each of the very close members of the Rho family which may indicate different functions for each protein.

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FIRST SESSION:
STRUCTURAL STUDIES ON G PROTEINS

Chairman: Alfred Wittinghofer

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ELONGATION FACTOR AS A MODEL FOR OTHER G-PROTEINS

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The general aim of our research programme is to study how alterations in a protein's structure can modify its biological function. The protein selected for study is the elongation factor EF-Tu. The long term goal of our research programme is to study this factor with special reference to modification of its substrate specificity, its interactions with EF-Ts and aa-tRNA, its sensitivity towards antibiotics and its thermal stability [1,2].

We believe that GTP and GDP act as allosteric effectors on their target proteins, thus altering the conformation. To understand what is happening at the molecular level, we need to know the three-dimensional structures of the particular protein-cofactor complexes. This necessitates X-ray crystallographic analyses which demand large amounts of protein produced by genetic engineering methods.

The 3-D structures of EF-Tu:GDP and EF-Tu:GTP have been determined by X-ray crystallography. A high resolution well refined electron density map of EF-Tu has enabled us and others to locate functionally important residues in the three-dimensional structure [3,4,5].

The structure is conveniently described in terms of three domains I, II and III which differ in contents of amounts and types of secondary structure. Domains I, II and III contain residues 1-199, 209-295 and 301-393, respectively. The structure of the GDP form is noteworthy for containing a hole in it.

The GDP/GTP molecule binds to the surface of domain I, also called the G-domain, which has a typical α/β type structure.

To date, we and our collaborators have succeeded in introducing mutations around the nucleotide binding site and in areas which are likely to be involved in interaction with tRNA, EF-Ts and the ribosome. We have also isolated the nucleotide binding domain, by introducing a large deletion in the gene, and shown that this new protein can still bind GDP.

Extensive comparison of translational factor sequences has shown that certain common elements of these sequences are retained. These fall into two classes: regions of general homology, stretching over one hundred amino acids or more, and particular groupings of amino-acid residues.

The loops involved in the 3-D structure defining the GDP binding site correspond to regions in primary sequences which show a high degree of homology when compared with other prokaryotic and eukaryotic elongation factors and initiation factor. The purine nucleotides play a

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crucial role as regulators of molecular functions and as sources of energy in protein biosynthesis. Guanosine di- and triphosphates are important as cofactors for elongation factors EF-Tu and EF-G and initiation factor IF2. Because of the high sequence conservation it has been possible to build convincing models based on the EF-Tu structure for other prokaryotic EF-Tu's such as *E. gracilis* EF-Tu, the eukaryotic EF-Tu (EF-1α) and prokaryotic IF-2.

During the transition from the GDP (inactive) to the GTP (active) form, domain 1 of EF-Tu, containing the GTP-binding site undergoes internal conformational change of the molecular switch similar to those observed in ras p21. In addition, a dramatic rearrangement of domains is observed, corresponding to a rotation of 90.8° of domain 1 relative to domains 2 and 3. Residues that are affected in the binding of aminoacyl-tRNA are found in or near the cleft formed by the domain interface. The recent determination of the structure of an intact form of *E. coli* EF-Tu GDP confirms that the conformational change is not affected by the nicked form of the protein.

After successful crystallization of the ternary complex [7] an X-ray crystallographic analysis [8] has shown that it has an extended shape of approximately 115Å in the longest dimension. The EF-Tu: GDPNP component binds exclusively to the acceptor arm of the Phe-tRNA, involving all three domains of the protein. The 3'-CCA end of the Phe-tRNA binds in a cleft formed by domains 1 and 2. In the GDP conformation of EF-Tu, a large conformational change takes place, destroying the binding site, and the factor is no longer capable of binding tRNA.

The action of aminoacyl-tRNA as a cofactor for the ribosome in stimulating GTP hydrolysis by EF-Tu can be assigned to the effector region of EF-Tu in good correlation of GTPase stimulators acting on effector regions of other GTP binding proteins. Details of such macromolecular interactions will be presented.

The structure of the ternary complex has been observed to be very similar to the structure of EF-G [9, 10] thus giving the first structural evidence supporting molecular mimicry of protein and RNA.

References

1. Riis, B. *et al.* (1990) Trends Biochem. Sci. 15, 420-424.
2. B.F.C. Clark, B.F.C. *et al.* (1995) In: tRNA: Structure, Biosynthesis and Function (eds. D. Söll & T. RajBhandary), American Society for Microbiology, Washington D.C. Ch. 21, pp. 423-442.
3. Kjeldgaard, M. & Nyborg, J.(1992) J. Mol. Biol. 223, 721-742.
4. Berchtold, H. *et al.* Nature (1993) 365, 126-132.
5. Kjeldgaard, M. *et al.* (1993) Structure 1, 35-50.
6. Wittinghofer, A. & Pai,E. (1991) TIBS 16,382-387.
7. Nissen *et al.* (1994) FEBS Letters, 356, 165-168
8. Nissen *et al.* (1995) Science, submitted.
9. Czworkowski *et al.* (1994). EMBO J. 13, 3661-3668.
10. Ævarsson *et al.* (1994) EMBO J. 13, 3669-3677.

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Structural studies on elongation factor Tu

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EF-Tu was the first protein found to be regulated by binding of GTP and GDP. In its active state, switched on by GTP binding, it forms a ternary complex with aminoacyl-tRNA and transports it to the A site of the messenger RNA-programmed ribosome, whereas subsequent GTP hydrolysis leads to the release of EF-Tu*GDP from the protein synthesis machinery. In the GDP form, the affinity of the EF-Tu to aminoacyl-tRNA is greatly reduced and the exchange of GDP for GTP is hindered by the slow dissociation rate of GDP from the complex. The guanine nucleotide exchange factor, EF-Ts, accelerates this dissociation and, owing to the high cellular GTP concentration, EF-Tu is retransformed back into its active GTP-bound form. Thus, EF-Tu cycles between the active GTP-bound state and the inactive GDP form, resembling other guanine nucleotide-binding proteins such as the heterotrimeric mammalian G proteins and the members of the ras protein family. The conformational differences between the inactive and the active forms are important for the molecular mechanisms of these signal-transducing GTPases [1].

We have determined the three-dimensional structure of the active GTP form of the EF-Tu from *Thermus thermophilus* at very high resolution (better than 1.7 Å), and compared it to the inactive, GDP-bound form [2]. When GDP is exchanged for GTP, the protein undergoes dramatic conformational rearrangements, with an amplitude of up to 42 Å. On the basis of the crystal structure, we propose molecular mechanisms for transduction and amplification of the signal induced by GTP binding as well as for the intrinsic and effector-enhanced GTPase activity of EF-Tu. These are supported by crystallographic studies of designed EF-Tu mutants [3].

The dramatic structural changes connected with nucleotide exchange serve the purpose of forming a specific binding cleft for aminoacyl-tRNA in the active GTP form but not in the inactive GDP form of EF-Tu [2]. We have determined the crystal structure of a complex between EF-Tu*GppNHp and anthraniloyl adenosine, which can be regarded as a model for the aminoacyl end of the tRNA. The analogue is found in the proposed tRNA binding cleft, with well-defined interactions with the protein.

When the antibiotic kirromycin binds to EF-Tu, the factor shows significant affinity to aminoacyl-tRNA not only in the GTP but also in the GDP form. Kirromycin is known to inhibit the release of EF-Tu*GDP from the ribosome after GTP hydrolysis. This prevents the peptidyl transferase reaction and subsequent translocation, and the ribosome is stalled [4]. Four mutations capable of conferring kirromycin-resistance to *E. coli* EF-Tu have been identified; all of them are located in the domain 1-3 interface of the GTP form of EF-Tu [5]. As this interface is completely remodelled upon GTP hydrolysis, it is understandble how kirromycin binding can interfere with the conformational rearrangement of the domains, leading to a breakdown of the allosteric control of EF-Tu in its interactions with tRNA and the ribosome.

Pulvomycin is another antibiotic interfering with the normal functions of EF-Tu by disrupting its allosteric control mechanisms. Again, we could locate pulvomycin-resistance mutations in an interface between EF-Tu domains; however, in this case the location identified is the point in the EF-Tu structure where all three domains meet [6].

Kirromycin and pulvomycin therefore represent drugs which do not act by binding to an active site but rather by preventing conformational rearrangements occuring during the function of their target, *i.e.* they behave like sand in the gear of the molecular switch, EF-Tu. Since antibiotic-sensitive EF-Tus are confined to the bacterial world, this protein with its essential role in bacterial polypeptide

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synthesis should be a most attractive target for new antibacterial drugs, be they rationally designed on the basis of our crystal structures or discovered by screening or combinatorial chemistry.

References:

[1] R. Hilgenfeld: *Nature Struct. Biol.* **2**, 3-6 (1995).

[2] H. Berchtold, L. Reshetnikova, C.O.A. Reiser, N.K. Schirmer, M. Sprinzl & R. Hilgenfeld: *Nature* **365**, 126-132 (1993).

[3] A. Wagner, J.R. Mesters, W. Zeidler, S. Ribeiro, M. Sprinzl & R. Hilgenfeld: *Biol. Chem. Hoppe-Seyler* **376**, 165 (1995).

[4] A. Parmeggiani & G.W.M. Swart: *Annu. Rev. Microbiol.* **39**, 557-577 (1985).

[5] J.R. Mesters, L.A.H. Zeef, R. Hilgenfeld, J.M. de Graaf, B. Kraal & L. Bosch: *EMBO J.* **13**, 4877-4885 (1994).

[6] L.A.H. Zeef, L. Bosch, P.H. Anborgh, R. Cetin, A. Parmeggiani & R. Hilgenfeld: *EMBO J.* **13**, 5113-5120 (1994)..

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Elongation factors from *Thermus thermophilus*

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Elongation factors Tu, Ts, and G from *T. thermophilus* were overproduced in *E. coli*, isolated and used for biochemical and structural studies. The crystal structures of EF-Tu·GppNHp, EF-Tu·GppC$_2$Hp and EF-Tu·GppNHp·anthranyloyl-adenosin have been solved in cooperation with Dr. R. Hilgenfeld (Jena). Crystals suitable for X-ray structure analysis were obtained from several mutants of EF-Tu, EF-Ts, EF-Tu·EF-Ts, valine-tRNA·EF-Tu·GppNHp complexes. The work on determination of these structures is in progress. Site-directed mutagenesis is used to gain information about mechanism of EF-Tu function in respect to GTPase, binding of aminoacyl-tRNA and interaction with ribosome. Amino acid replacements were performed on Glu55, Glu56, Arg59, Thr62, His67, Asp81, Cys82, His85, Gly233, Arg300 and Thr394. Deletion mutants were 181-190, 212-405, 313-405, 1-316, and 212-405. Properties of these EF-Tu variants will be discussed. Aminoacyl-tRNA binds to EF-Tu in form of an amino acid orthoester. The formation of this structure is necessary for stability of aminoacyl-tRNA·EF-Tu·GTP ternary complexes. Analogues of an orthoester form of amino acid are inhibitors of protein biosynthesis. A model explaining the mode of action of chloramphenicol will be presented.

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Structure of the human ADP-ribosylation factor

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ADP-ribosylation factors (ARFs) are essential and ubiquitous in eukaryotes. They are involved in vesicular transport, and function as activators of phopholipase D and cholera toxin. The activity of ARF proteins in membrane traffic and organelle integrity are intimately tied to its reversible association with membranes and specific interactions with membrane phospholipids. One common feature of these functions is their regulation by the binding and hydrolysis of GTP.
Sequence comparisons show that the ARF molecule is similar to other GTP binding proteins, such as ras family members, EF-Tu and the nucleotide binding domain of the a-subunit of the G protein transducin. However, there are some structural differences that differentiate ARF from these proteins, in that it has an N-terminal extension that is essential to ARF function and has no counterpart in other GTP-binding proteins. The N-terminal glycine is myristoylated and may serve as an anchor when the protein interacts with the membrane.

We have solved the three-dimensional structure of human ARF1 in the GDP-bound, non-myristoylated form. This is a soluble form of the protein that crystallizes readily. The structure has been used to interpret some of the characteristics of ARF, namely, the GTP-dependent modulation of membrane affinity, the lack of intrinsic GTPase activity, and the nature of the effector binding surfaces.

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MUTAGENIC ANALYSIS OF α–GUSTDUCIN DOMAINS INVOLVED IN INTERACTION WITH BITTER TASTE RECEPTORS.

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Gustducin is a taste-specific G protein of the transducin family involved in bitter signal transduction in taste receptor cells. We have characterized a membrane fraction from bovine and mice taste tissue that activates transducin and gustducin upon stimulation with the bitter compound denatonium. In order to further characterize this receptor activity, we have mutated two residues on α-gustducin located in receptor interaction domains already described for other G proteins. Mutations in cys^{324} (C^{324}-->A^{324}, C^{324}-->S^{324}) didn't affect the ability of the proteins to be activated by the heterologous receptor rhodopsin, nor affected the activation of the gustducin mutants upon denatonium stimulation of taste membranes. These mutations reduced the affinity to GDP and changed the tryptic sensitivity of the mutants in a way that can be explained by assigning cys^{324} a major role in maintaining the non active, GDP-bound conformation of gustducin in the absence of activated receptor. Mutation in gly^{351} (G^{351}-->P^{351}), a mutation that should provoke a major conformational change in the proposed structure of the C-terminal end of gustducin, blocked completely both the rhodopsin activation and the denatonium-mediated taste-membrane activation of the G^{351}-->P^{351} mutant. It is concluded that the denatonium activation of gustducin in taste membranes occurs upon stimulation of a taste receptor that interacts with gustducin in a similar way to the rhodopsin-transducin interaction. L. R-A is a Fulbright scholar from the Spanish MEC-Fulbright postdoctoral program.

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Interaction of the retinal cyclic GMP phosphodiesterase inhibitor with transducin ($G_o\alpha$) and with other G-Proteins ($G_i\alpha$, $G_o\alpha$ and $G_s\alpha$)

Vertebrate visual excitation is mediated by a light-triggered G-protein cascade. In the retinal cyclic GMP-phosphodiesterase (PDE), catalysis by the αβ-heterodimer is inhibited in the dark by two identical γ-subunits and stimulated in the light by the GTP-bearing α-subunit of the heterotrimeric G-protein transducin (Tβγ-TαGDP). Two TαGTP molecules, dissociated from Tβγ, bind to and displace the PDEγ subunits from their inhibitory sites on PDEαβ. We have studied the interaction of recombinant bovine PDEγ with purified Tα in solution. The kinetics and affinity of this interaction were determined by monitoring changes in the proteins' tryptophan fluorescence. The K_d's for the binding of recombinant PDEγ to soluble TαGTPγS and TαGDP are ≤ 0.1 nM and 3 nM, respectively. Site-directed mutagenesis shows that both W207 of Tα and W70 of PDEγ are crucial for this interaction.

In addition we observe that while PDEγ is not the physiological effector of other Gα subtypes, it can still detectably interact with them. This interaction is strong with $G_{i1}\alpha$ and $G_{i3}\alpha$ ($K_D \approx 10$ nM) and weaker with $G_0\alpha$ and $G_s\alpha$ ($K_D \approx 1$ mM). For all these Gα subtypes, similar intrinsic fluorescence changes are observed upon PDEγ binding. Moreover, similar relative decreases in affinity are obtained when the GDP forms of $G_{i1}\alpha$, $G_{i3}\alpha$ or $G_t\alpha$ are used in lieu of the GTP forms. This points to a conserved GTP-dependent effector-interaction domain which includes probably the conserved W (207 in Tα) of the switch II domain.

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SECOND SESSION:
STRUCTURE AND MECHANISMS OF ACTION OF G PROTEINS

Chairman: Brian F. C. Clark

Instituto Juan March (Madrid)

G proteins: a conserved structure for multiple functions.

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Along the last years we have seen a fantastic increase in the number of known sequences belonging to thedifferent families of G proteins, i.e. ras, EF-Tu, G protein alpha subunit, arf. Updated versions of the phylogenetic trees of each one of the families will be discussed. The analysis will be done with a particular emphasis on the influence of the different sequencing projects (Heamophilus influenzae, Mycoplasma genitalium, Saccharomyces cerevisiae) on our understanding of the superfamily.

Parallel to the efforts in sequencing the structure of different proteins of the superfamily has been solved (ras-p21, EF-Tu, EF-G, G protein alpha subunit, arf). Comparison of the available structures becomes a powerful tool for the deciphering of differences and similarities in the action mechanisms. Specially relevant are the different conformational changes promoted by the GTP hydrolysis in different systems.

Finally, the use of the newly available tools in sequence-structure analysis leads to two pictures:

- A description of the elements common to all G proteins. These regions are related with the common GTP binding function and

- a proposal about the key regions creating the functional differences between different families of the G protein superfamily.

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Tubulin: a GTP binding protein

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In addition to the existence of several small G proteins with a regulatory function on cell growth, there is an ubiquitous structural GTP binding protein that plays an important role in the regulation of cell division, tubulin. Tubulin is a protein whose quaternary structure is a heterodimer formed by two subunits (α and ß) which requires of chaperon-like proteins to fold in a proper functional way. Once the heterodimer has been assembled, tubulin function, inside the cell, is based in its capacity to polymerize (into microtubules) or to depolymerize back into heterodimers, in a dynamic way. In that dynamic process, GTP binding to tubulin is required for microtubule assembly and GTP hydrolysis for microtubule disassembly. Two GTP binding sites are present in tubulin heterodimer. One exchangeable with exogenous GTP, and present in the ß subunit, and the other, which is non exchangeable, present in α subunit.

The exchangeable GTP bound to ß subunit becames non exchangeable once it is incorporated into the microtubule and, in the polymer, it hydrolyzes yielding GDP. This hydrolysis is not totally coupled to the assembly.

Thus, as previously indicated, GTP binding to tubulin is needed for microtubule polymerization, being GTP hydrolysis required for microtubule depolymerization, and it is possible to pass from a net assembly of tubulin into microtubules (growing state) to a fast depolymerization (shrinking state) when the number of tubulin subunits, present in the polymer, containing GTP bound to them, decreases below a threshold, and mainly the microtubule is composed of GDP-tubulin.

On the other hand, in the presence of some microtubule associated proteins, MAPs, microtubules became more stable and less dynamic structures.

In this communication two aspects will be considered: the possible influence of microtubule associated proteins (MAPs) in the GTP binding to tubulin and the folding of the GTP binding site. For the first point it was observed that the presence of MAPs facilitate GTP binding. For the second point it was observed that the formation of GTP binding loop, identified by doing point mutations on beta tubulin molecule, probably could be required for the further proper folding of beta tubulin.

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The Structure and Mechanism of Heterotrimeric G-proteins

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Thousands of different extracellular signals are transmitted to their intracellular targets ('effectors') through 7-transmembrane helical receptors and their cognate heterotrimeric G-proteins. Because of the availability of adequate amounts of the relevant proteins and the extensive body of biochemical and biophysical research, we have studied the visual response of the bovine retina. The heterotrimeric G-protein is called transducin or $G_{t\alpha\beta\gamma}$.

Four crystal structures have been solved to 2.2 Å or better. $G_{t\alpha}$·GDP when compared to $G_{t\alpha}$·GTPγS reveals the stereochemical determinants of "activation" of the α-subunit. $G_{t\alpha}$·GDP·AlF_4 is a transition-state mimic for the hydrolysis of GTP and, thereby, gives strong insight into the mechanism. $G_{t\beta\gamma}$ gives a vivid picture of the structure of the βγ-component that stabilizes the inactive state of G_α and in certain systems serves as a signal transducer. Finally, the structure of $G_{t\alpha\beta\gamma}$·GDP when contrasted with the above shows the mechanism of disengagement of $G_{\beta\gamma}$ from G_α·GTP upon activation. What remains is to establish the stereochemistry that defines the mechanism by which the photo-activated rhodopsin catalyzes the replacement of GDP with GTP and the activating interaction between $G_{t\alpha}$·GTP and the γ-subunit of its effector, cGMP phosphodiesterase.

References:

Noel, J.P., Hamm, H.E. and Sigler, P.B. (1993). The 2.2 Å crystal structure of transducin α·GTPγS. *Nature* , **366,** 654-663.

Lambright, D.L., Noel, J.P., Hamm, H.E. and Sigler, P.B. (1994). The 1.8 Å crystal structure of transducin α·GDP: Structural Determinants for Activation of a heterotrimeric G-protein α subunit. *Nature*, **369**, 621-628.

Sondek, J., Lambright, D.G., Noel, J.P., Heidi E. Hamm, and Sigler, P.B. (1994). The 1.7 Å crystal structure of transducin α·GDP·AlF_4^- reveals the mechanism of GTP hydrolysis. *Nature*, **372,** 276-279.

Instituto Juan March (Madrid)

Structural and Functional Studies on the Ras-Raf Interaction

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The oncogenproduct p21^{ras} is involved in the transduction of signals from the plasma membrane to the nucleus. It is a GTP-binding protein with functions as a switch between the GTP-bound On-state and the GDP-bound OFF-state. Its interaction with the regulatory region of the downstream effector, the protein kinase c-Raf-1 has been investigated biochemically and structurally. A method has been developed to measure this interaction biochemically which is based on the fact that the Ras-Binding Domain of c-Raf-1 acts as a GDI, an inhibitor of nucleotide dissociation. The three-dimensional structure of p21^{ras} and its antagonist p21rap1A bound to the RAS-Binding-Domain of the Raf have solved by X-ray crystallography to high resolution. The details of this interaction will be described.

References

Emerson, S.D. et al., *Biochemistry* **33**, 7745-7752 (1994)
Nassar, C. et al., in Vorbereitung
van Aelst, L., et al., *Proc. Natl. Acad. Sci. USA,* **90**, 6213-6219 (1993)
Vojtek, A.B., Hollenberg, S.M., and Cooper, J.A., *Cell* **74**, 205-214 (1993)
Warne, P.H., Rodriguez Viciana, P., and Downward, J., *Nature* **364**, 352-355 (1993)
Zhang, X.-f. et al., *Nature* **364**, 308-313 (1993)

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Specificity of phosphotyrosine peptide binding to the SH2 domain of Grb2, an adaptor protein involved in the activation of ras pathway.

Grb2 is an 'adaptor' protein made exclusively of one SH2 domain flanked by two SH3 domains. Grb2 forms a stable complex with the ras exchange factor Sos, the SH3 domains of Grb2 interacting with proline rich motifs in the C-terminal of Sos. Binding of the Grb2 SH2 domain on receptors, or other adaptor proteins such as Shc, recruits this Grb2/Sos complex at the plasma membrane where Sos stimulates nucleotide exchange on ras, then ras activates raf and leads to MAP-kinase activation.

We have used tryptophan fluorescence changes to study the specificity and affinity of phosphotyrosine peptide binding to the SH2 domain of Grb2. An asparagine residue in Y+2 (**p**Y̲XNX) had been showed to be the consensus sequence recognised by this SH2 domain. We have thus tested different phosphotyrosine peptides derived from several receptors and adaptors. The Shc peptide (PS**p**Y̲V**N**VQN named P8N) possesses the highest affinity. Substitutions of the asparagine (**N**) decrease the affinity by at least two order of magnitude. Intensity of fluorescence and emission spectra are also changed showing that the **N** in Y+2 interacts with the tryptophan 121 present in the SH2 domain. Using phosphotyrosine peptides from P8N with sequential changings of amino acids in Y-1, Y+3, Y+4 and Y+5 allow us also to determine the contribution of these residues in the binding. However some peptides with an aspartate (**D**) in Y+2 also bind Grb2 with reasonable affinities (µM range) suggesting that some proteins that can be phosphorylated on tyrosine, but do not possess the **p**Y̲XNX consensus motif may interact with the SH2 domain of Grb2 and thus lead to the activation of the ras pathway.

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THIRD SESSION: INVOLVEMENT OF G PROTEINS IN CELL FUNCTION (I)

Chairman: Yoshimi Takai

Instituto Juan March (Madrid)

G12 requirement for thrombin-stimulated gene expression and DNA synthesis in 1321N1 astrocytoma cells

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Thrombin is a potent mitogen for fibroblasts, astrocytes and other cell lines (Van Obberghen-Schilling et al., 1985; Cavanaugh et al., 1990; Hung et al., 1992 and LaMorte et al., 1993a; 1993b). Thrombin cleaves and activates a seven-transmembrane spanning receptor to trigger G-protein mediated stimulation of downstream effectors (Vu et al., 1991). The actions of thrombin in 1321N1 human astrocytoma cells have been well characterized. In these cells, thrombin stimulates phospholipase C activity leading to mobilization of intracellular calcium, diglyceride generation and redistribution of protein kinase C (Jones et al, 1989; Nieto et al., 1994; Post et al., submitted). Thrombin receptor activation also leads to a biphasic increase in c-jun mRNA, an associated increase in AP-1 DNA binding activity and a marked increase in AP-1-mediated gene expression and DNA synthesis (Trejo et al., 1992; LaMorte et al., 1993b). Ras function is required for the mitogenic effect of thrombin as well as thrombin induced AP-1 transcriptional activity (LaMorte et al., 1993b).

There is substantial information about the signaling pathways downstream of thrombin receptor activation; however, it is not known which of the heterotrimeric G proteins couples thrombin to the mitogenic pathway. In fibroblasts, thrombin stimulated DNA synthesis is sensitive to pertussis toxin (PTX) (Chambard et al., 1987; van Corven et al., 1993). Experiments using microinjected antibodies against G_o or G_i suggested that the mitogenic effect of thrombin in CCL39 and in 3T3 fibroblasts is mediated through these G proteins (LaMorte et al., 1993a; Baffy et al., 1994). However in 1321N1 astrocytoma cells, thrombin stimulated DNA synthesis and thrombin-induced gene expression are not inhibited by pertussis toxin treatment (Post et al., submitted) suggesting that G proteins of the G_i/G_o family do not mediate these responses. The PTX insensitive G proteins Gq and/or G11 have also been reported to function in mitogenic signaling by thrombin and bradykinin in fibroblasts (LaMorte et al., 1993a; Baffy et al., 1994). However, studies comparing muscarinic and thrombin receptor signaling mechanisms in astrocytes and CCL39 fibroblasts demonstrate that, although both receptors interact with Gq/G11 to stimulate phospholipase C, release calcium and activate PKC, only thrombin can activate Ras and elicit cell proliferation (Trejo et al., 1992; Seuwen et al, 1990; Post et al., submitted). These data suggest that activation of Gq and phospholipase C is insufficient to account for the full mitogenic effects of thrombin and that other signaling pathways are involved.

The Gα12 and Gα13 subunits constitute a family of G proteins distantly related to the other G protein a subunits (Strathmann and Simon, 1991). Both Gα12 and Gα13 lack the cysteine residue which renders these proteins susceptible to ADP-ribosylation by PTX. The signaling pathways regulated by these G proteins have not been identified. Activation of these G proteins has nonetheless been linked to cell growth in several cell systems.
Overexpression of Gα12 in NIH3T3 cells leads to neoplastic transformation (Chan et al, 1993) and expression of a GTPase- deficient mutant form of Gα12 and Gα13 can efficiently transform NIH3T3 cells (Jiang et al, 1993; Xu et al, 1993) and Rat-1 fibroblasts (Voyno-Yasenetskaya et al, 1994a). In addition, EGF- stimulated MAPK activity is enhanced in fibroblasts that express the activated mutants of Gα12 and Gα13 (Voyno-Yasenetskaya et al., 1994b). Therefore these G proteins appear to be good candidates to be involved in PTX-insensitive signaling pathways leading to cell growth.

In the present study we show that the activated mutant form of Gα12 induces AP-1 mediated transcriptional activation when transfected in 1321N1 cells and this effect requires Ras function. Furthermore, we show that microinjection of an antibody against Gα12 can specifically block the mitogenic effect of thrombin. Taken together, these results suggest that Gα12 functions as a PTX-insensitive mediator of thrombin induced DNA synthesis in 1321N1 cells.

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References

Baffy, G., Yang, L., Raj, S., Manning, D. R. and Williamson, J. R. (1994) J. Biol. Chem. 269, 8483-8487.
Cavanaugh, K. P., Gurwitz, D., Cunningham, D., D. and Bradshaw, R., A. (1990) J.
Chambard, J., C., Paris, S., L'Allemain, G. and Pouyssegur, J. (1987) Nature 326, 800-803.
Chan, A., M., -L., Fleming, T., P., McGovern, E. S., Chedid, M. , Miki. T. and Aaronson, S. A. (1993) Molecular and Cellular Biology 13, 762- 768.
Hung, D., T., Vu, T., H., Nelken, N., A. and Coughlin, S., R. (1992) J. Cell Biol. 116, 827-832.
Jiang, H., Wu, D. and Simon, M., I. (1993) FEBS 330, 319-322.
Jones, L., G., McDonough, P., M. and Brown, J., H. (1989) Mol. Pharmacol. 36, 142-149.
Koch, W. J., Hawes, B., E., Allen, L., F. and Lefkowitz, R., J. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 12706-12710.
LaMorte, V., J., Harootunian, A., T., Spiegel, A., M. , Tsien, R., Y. and Feramisco, J., R. (1993a) J. Cell Biol. 121, 91-99.
LaMorte, V., J., Kennedy, E., D. , Collins, L., R. , Goldstein, D. , Harootunian, A. T. , Brown, J., H. and Feramisco, J., R. (1993b) J. Biol. Chem. 268, 19411-19415.
Nieto, M., Kennedy, E., Goldstein, D. and Brown, J., H. (1994) Mol. Pharmacol. 46, 406-413.
Offermanns, S., Laugwitz, K-., L., Spicher, K. and Schultz, G. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 504-508.
Post, G., R., Collins, L. R. , Kennedy, E., D. , Aragay, A., M., Goldstein, D. and Brown, J. H. (1995) Submitted.
Seuwen, K., Cahan, C. , Hartmann, T. and Pouyssegur, J. (1990) J. Biol. Chem. 265, 22292-22299.
Strathmann, M., P. and Simon, M., I. (1991) Proc. Natl. Aca. Sci. U.S.A. 88, 5582-5586.
Trejo, J. , Chambard, J-., C. , Karin, M. and Brown, J., H. (1992) Mol. Cel. Biol. 12, 4742-4750.
Van Corven, E., J., Hordijk, P., L., Medema, R. H., Bos, J., L. and Moolenaar, W. H. (1993) Neurochem. 54, 1735-1743.
Van Obberghen-Schilling, E., Chambard, J., C., Paris, S., Alleman, G., L. and Pouyssegur, J. (1985) EMBO J. 4, 2927-2832.
Voyno-Yasenetskaya, T., A., Conklin, B., R.,Gilbert, R., L., Hooley, R., Bourne, H., R. and Barber, D., L. (1994a) J. Biol. Chem. 269, 4721- 4724.
Voyno-Yasenetskaya, T., A., Pace, A., M. and Bourne, H., R. (1994b) Oncogene 9, 2559-2565.
Vu, T.,-K., H., Hung, D., T., Wheaton, V., I. and Coughlin, S., R. (1991) Cell 64, 1057-1068.
Xu, N., Bradley, L. , Ambdukar, I. and Gutkind. J., S. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 6741-6745.

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THROMBIN RECEPTOR-INDUCED MITOGENIC SIGNALS

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The serine protease thrombin (THR) elicits a biological response in a wide variety of cells, including growth stimulation in vascular smooth muscle cells and fibroblasts. Thrombin-responsive CCL39 cells, a line of Chinese hamster lung fibroblasts, led us to dissect THR-induced mitogenic signals. In these cells, THR, like agonists for receptor tyrosine kinases, activates persistently the MAP kinase (p42/p44) cascade, a pathway that is obligatory to induce cell cycle entry. THR activates this pathway by cleaving its 7 transmembrane domain receptor, a step that leads to dissociation of multiple heterotrimeric G proteins: Gq, G_{12} and the Pertussis toxin-sensitive Gi/o. We showed that these G proteins activate cytoplasmic tyrosine kinases, including members of the Src family, Src and Fyn. We believe that rapid THR-induced tyrosine phosphorylation events are essential to couple the THR receptor to Ras. Indeed we found that THR rapidly stimulates Shc phosphorylation on tyrosine, an action that promotes recruitment of the complex Grb2/Sos. THR-stimulated MAP kinase activity and cell proliferation are stimulated by overexpression of Shc, and strongly inhibited by the expression of the Shc tyrosine mutant, Y317F, deficient in Grb2 binding. These results indicate that Shc plays a critical role in the transmission of mitogenic signals whether they emerge from receptor tyrosine kinases or from G protein-coupled receptors.

In addition, we demonstrate that the tyrosine phosphatase PTP1D plays a positive role in the THR-induced mitogenic response, like it was shown for the PDGF receptor. We therefore conclude that THR receptor activation is coupled to the Ras/MAP kinase cascade *via* at least two independent signalling molecules, Shc and PTP1D.

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MECHANISM OF ACTION OF RAS REGULATORS

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The GDP/GTP cycle of ras gene products is regulated by two kinds of ligands, the GTPase activating proteins (GAPs), that by removing the GTP-bound active state of ras negatively influence the downstream signal, and the GDP/GTP exchange factors (GEFs) that regenerate the active complex. To study their mechanism of action *in vitro*, we have overproduced the domains of GAPs and GEFs from mammals and yeast *S. cerevisiae* using *E. coli* as host cell. By means of several chromatographic methods we have isolated these products in part in highly purified form. We have studied the kinetic properties of the action of the catalytic domain of Ira2p, a GAP protein from *S. cerevisiae*, on Ras2p activity and its capacity to interact with tubulin as compared to mammalian p120-GAP and neurofibromin. A remarkable and as yet unexplained property is the selectivity of the action of Ira proteins. In fact, the ras proteins and their regulators GAP and GEF of mammals and *S. cerevisiae* display structural similarities and are functionally interchangeable *in vivo* and *in vitro* with the only exception of Ira1p and Ira2p that are strictly specific for *S. cerevisiae* Ras proteins and cannot stimulate the GTPase of mammalian H-ras p21. We have investigated the reason of this specificity by constructing a series of H-ras/Ras2p chimaeras and mutated ras proteins and were able to identify structural determinants of ras responsible for the selective action of Ira2p. The results help clarify specific aspects of the conserved molecular mechanisms of the interaction between ras proteins and their GAPs.

Concerning the GDP/GTP exchangers, we have characterized several aspects of the interaction between H-ras and the catalytic domain of the mammalian brain-specific GEF $CDC25^{Mm}$, isolated in pure form. The formation of its stable complex obtained with nucleotide-free H-ras was analyzed by electrophoretic and chromatographic techniques. Several kinetic properties of the $CDC25^{Mm}$ interaction with H-ras p21 were investigated. In particular, the apparent affinity of the nucleotide-free p21•C-$CDC25^{Mm}$ complex and whether the nature of the ras-bound and of the free nucleotide affect the rate of the C-$CDC25^{Mm}$-mediated nucleotide exchange on p21. The results were compared with those obtained with other GEFs, from which C-$CDC25^{Mm}$ differs for a number of specific properties. Also the influence of the N-terminal domain on the activity of the catalytic domain of $CDC25^{Mm}$ was examined under a number of experimental conditions.

Parrini, M.C., Jacquet, E., Bernardi, A., Jacquet, M. & Parmeggiani, A.
Properties and Regulation of the Catalytic Domain of Ira2p a *Saccharomyces cerevisiae* GTPase Activating Protein of Ras2p
Biochemistry (1995), in print

Jacquet, E., Baouz, S. & Parmeggiani, A.
Characterization of Mammalian C-$CDC25^{Mm}$ Exchange Factor and Kinetic Properties of the Exchange Reaction Intermediate p21•C-$CDC25^{Mm}$
Biochemistry (1995), in print

Instituto Juan March (Madrid)

Berthe Marie Willumsen:

THE RAS PROTEIN: SITES OF INTERACTION WITH EFFECTORS AND STIMULATORS

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We have investigated the requirement for particular sequences for the H-Ras protein using transformation as biological assay. Our results have led to a detailed understanding of the Ras protein and the interactions it must provide in order to function.

The extreme C-terminus is involved anchoring the protein in the membrane. The membrane localization is effected by several posttranslational modifications including, for those Ras proteins containing additional cysteines within 4-8 residues, palmitoylation of these. We have shown that interference with palmitoylation significantly reduces the transforming activity of the activated *ras* gene. We find, however, that the C-terminus and its modifications are dispensable for transforming activity, since membrane association via myristoylation of a premature truncation mutant restores transforming activity. In addition, we find that sequences close to the palmitoylated cysteines influence the membrane association of lipid modified Ras protein, suggesting a previously unrecognized signal of importance for Ras activity.

We have analyzed the sequence requirements for transforming activity of both activated and normal H-*ras* genes, and this way have mapped regions that need to be intact for effector function, for GTPase acceleration by GAP and for stimulation by the exchange protein GRF, the protein homologous to the ras activator of *S. cerevisiae* CDC25. We have also investigated the Ras exchange proteins Sos1 and Sos2 for biological activity. Membrane association via a myristoylation signal changes Sos1 to a transforming protein; however, the Sos2 protein seems to have much lower activity. We have explored the basis for this defect, and suggest that the Sos2 protein, in contrast to Sos1, is subject to ubiquitin mediated degradation.

Segal, M., Marbach, I., Willumsen, B. M. and Levitzki, A. (1995): Two distinct regions of Ras participate in functional interaction with GDP-GTP exchangers. Eur. J. Biochem., *228*: 96-101.

Willumsen, B. M., Papageorge, A. G., Kung, H.-F., Bekesi, E., Robins, T., Johnsen, M., Vass, W. C. and Lowy, D. R. (1986): Mutational analysis of a *ras* catalytic domain. Mol. Cell. Biol., *6*: 2646-2654.

Willumsen, B. M., Vass, W. C., Velu, T. J., Papageorge, A. G., Schiller, J. and Lowy, D. R. (1991): The BPV E5 oncogene can cooperate with *ras*: identification of a p21 amino acid segment required for transformation by c-ras^H but not v-ras^H. Mol. Cell. Biol., *9*: 6026-6033.

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TWO DIFFERENTIALLY EXPRESSED hSos1 ISOFORMS WITH DIFFERENT GRB2 BINDING AFFINITY AND BIOLOGICAL ACTIVITY

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We compared structure, expression and functional properties of two hSos1 cDNA isoforms (Isf I and Isf II) isolated, respectively, from human fetal brain and adult skeletal muscle libraries. Isf I and Isf II nucleotide sequences differ only by the presence in Isf II of an in-frame 45 bp insertion located near the first proline-rich motif required for Grb2 binding. Some human tissues express only one isoform (fetal brain: Isf I; adult skeletal muscle: Isf II) while most others express variable proportions of both in fetal and adult stages. In vitro binding studies and in vivo assays (yeast two hybrid system and NIH 3T3 transfection assays) indicated that Isf II exhibits significantly higher Grb2 binding affinity and biological activity than Isf I. These results suggest that functionally different hSos1 isoforms with differential tissue expression and distribution, may contribute to finely regulated mechanisms controling Ras activation at the tissue and developmental stage level.

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IDENTIFICATION OF TUBERIN, THE *TSC2* TUMOR SUPPRESSOR GENE PRODUCT, AS A SPECFIC GAP FOR RAP1

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Tuberous sclerosis (TSC) is a genetic syndrome characterized by the development of benign tumors in a variety of tissues, as well as rare malignancies. Two different genetic loci have been implicated in TSC and appear to function as tumor suppresser genes; one of these loci, the tuberous sclerosis-2 gene (*TSC2*), encodes an open reading frame with a putative protein product (tuberin) of 1784 amino acids. Tuberin contains a region of limited homology to the catalytic domain of Rap1GAP near its C-terminus, suggesting that tuberin may function as a negative regulator of this small, Ras-related GTPase. Furthermore, predisposition to renal carcinoma of the Eker rat strain is determined by a heterozygous germ-line insertion within the *TSC2* gene, causing a premature stop codon 5' to the region with homology to Rap1GAP.

We have generated several antisera against the N-terminal and C-terminal portions of tuberin, and these antisera specifically recognize a 180-kDa protein in immunoprecipitation and immunoblotting analyses of cell extracts. Subcellular fractionation revealed that most tuberin is found in a membrane/particulate (100,000 x *g*) fraction. Immunofluorescence analysis demonstrated that the majority of tuberin is localized to the cis/medial Golgi, and the staining pattern was quite similar to that seen for Rap1, consistent with a possible interaction between these proteins *in vivo*. A wide variety of cell lines express the 180-kDa tuberin protein. Western-blot analyses of several cell lines derived from renal carcinomas of the Eker rat strain revealed a lack of tuberin in these cell lines, confirming a 'second hit mutation' at *TSC2* during the development of the malignancies. Additional evidence for a tumor suppressor role for *TSC2* was revealed by the loss of tuberin expression in several sporadic brain tumors fom non-TSC patients.

Immunoprecipitates of native tuberin from K-562 cells contain an activity that specifically stimulates the intrinsic GTPase activity of Rap1a, although there may be additional sources of Rap1GAP activity in this cell line. Rap1GAP activity was confirmed in assays with a C-terminal fragment of tuberin, expressed in bacteria or SF9 cells. Tuberin did not stimulate the GTPase activity of Rap2, Ha-Ras, Rac, Ran, or Rho. We conclude that tuberin is a specific Rap1GAP. These results provide experimental evidence for a striking parallel between TSC and neurofibromatosis type 1 (NF1), in which the loss of functional tuberin (which normally would inactivate Rap1) may lead to constitutive activation of Rap1 in tumors of patients with tuberous sclerosis. Furthermore, in the tuberin-deficient Eker cell lines, the lack of Rap1GAP activity of tuberin might be critical for their proliferation, analogous to the role of the *NF1* product neurofibromin, the crucial RasGAP in human Schwann cell tumors. We are currently exploring these possibilities.

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FOURTH SESSION: INVOLVEMENT OF G PROTEINS IN CELL FUNCTION (II)

Chairman: Johannes L. Bos

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Rho proteins function as dual regulators of cell growth and apoptosis

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Oncogenes appear to influence apoptosis in two ways. Some activate cells from a growth-arrested state to one in which both apoptosis and entry to S-phase become possible, the choice between them being determined by a second signal, such as cytokines or growth factors. Cells in this state are often sensitive to apoptosis induced by a wide variety of agents, including several drugs used in cancer chemotherapy. Other oncogenes prevent activation of the apoptosis effector pathway, even in the presence of a death stimulus, the affected cells therefore being resistant to chemotherapeutic agents. In rodent fibroblasts, c-*myc* or the adenovirus oncogene E1A effect the first type of change, whereas *bcl*-2, v-*abl*, E1B or activated *ras* effect the second.

We have demonstrated that *rho* genes, members of the *ras* superfamily, are tumorigenic when overexpressed in NIH-3T3 cells (1,2), and as other known oncogenes, they also induce apoptosis after serum deprivation (3). The apoptotic index in tumors generated by these cell lines is similar to those induced by E1A-transformed cells and much higher than those found in tumors induced by *ras*-transformed cells. Apoptosis induced by *rho* is blocked by overexpression of *blc*-2, and a drastic reduction in the apoptotic index was observed in tumors generated in nude mice by cell lines expressing both *rho* and *bcl-2*. Thus, *rho* genes may play a critical role in the regulation of apoptosis both *in vitro* and *in vivo*.

We will provide evidence that overexpression of Rho proteins in NIH 3T3 cells induce the generation of phosphatidylcholine (PC)-derived second messengers as a result of activation of a PC-specific phospholipase D (PC-PLD) as found for *ras*-transformed cells. In contrast, removal of serum in the Rho transfectants, but not in normal NIH 3T3 cells or cells transformed by the *ras* oncogene, induced the production of ceramides as a result of activation of an sphingomyelinase (SMase). Furthermore, the *rho*-expressing cells underwent apoptosis in the presence of serum when exogenous ceramides were added, and this process was accelerated if cells were treated with exogenous SMase. Thus, Rho proteins act as an initiation signal that is necessary but not sufficient for the induction of apoptosis in NIH 3T3 cells. We propose that induction of apoptosis in NIH 3T3 cells requires two complementary signals: an initiation signal generated even in

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the presence of serum which "primes" the cells, making them sensitive to a progression signal, triggered by serum removal, which we have identified as generation of ceramides.

Recently, it has been found that the Rho family of small GTPases are critical elements involved in the regulation of signal transduction cascades from extracellular stimuli to the cell nucleus. Both the JNK/SAPKs signaling pathway and the c-*fos* serum response element (SRF) are activated by members of the Rho family. We will provide evidence of a novel signaling pathway activated by the Rho proteins which may be responsible for some of their biological activities such as transformation, cytoskeleton organisation, apoptosis and metastasis. The human rho A, rho B, CDC42 and rac 1 proteins efficiently induce the transcriptional activity of nuclear factor κB (NF-κB) by a mechanism independent of the Ras GTPase and the Raf-1 kinase. This activation is reversed by overexpression of IκBa, the NF κB inhibitor, suggesting that Rho regulates NF κB activity by dissociation of the NF κB:IκBα complex.

Therefore, in addition to their transforming activity, expression of *rho* oncogenes, members of the *ras* superfamily induce apoptosis in rodent fibroblasts. Apoptosis induced by Rho requires two signals, one of them is ceramides production, which may be related to NF κB transcriptional activation in a Ras-independent pathway.

1.- **Ballestero, R.P., Esteve, P., Perona, R., Jiménez, B., Lacal, J.C. (1991)** Biological function of *Aplysia californica rho* gene. En The Superfamily of *ras* related Genes, pp. 237-242. NATO Advanced Science Institute Series, vol. A220. Plenum Press.

2.- **Perona R., Esteve P., Jimenez B., Ballestero R.P., and Lacal J.C. (1993)**. Tumorigenic activity of rho genes from Aplysia californica. Oncogene 8, 1285-1292.

3.- **Jiménez B., Arends M., Esteve P, Perona R., Sanchez R., Ramón y Cajal S, Wyllie A. and Lacal J.C. (1995)**. Induction of apoptosis in NIH3T3 cells after serum deprivation by overexpression of rho-p21, a GTPase protein of the ras superfamily. Oncogene 10, 811-816.

4.- **Esteve P., del Peso L. and Lacal J.C. (1995)** Induction of apoptosis by *rho* in NIH 3T3 cells requires two complementary signals. Ceramides function as a progression factor for apoptosis. Oncogene (in press).

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Title:

Signaling from the membrane to the nucleus: Ras and Rho-related GTP-binding proteins control independent kinase cascades communicating cell surface receptors to distinct members of the MAPK superfamily.

Abstract:

We have used the expression of human muscarinic acetylcholine receptors (mAChRs) in NIH 3T3 cells as a model for studying proliferative signaling through G protein-coupled receptors. In this biological system, the m1 class of mAChRs can effectively transduce mitogenic signals, and induce malignant transformation if persistently activated (1,2). Consistent with this observation was our finding that mutated genes for certain classes of G proteins are as transforming as the most potent known oncogenes (3,4). We then began dissecting biochemical pathways utilized by transforming G protein-coupled receptors. We found that one such pathway involves the exchange of GDP for GTP in the protein product of the *ras* proto-oncogene. The nucleotide exchange in p21^{ras} leads to the activation of the p72^{raf} kinase, and both events were found to be strictly required to induce DNA-synthesis or transformation by G protein-coupled receptors (5 and unpublished observations). Upon activation, p72^{raf} is believed to be one of the initiators of a cascade of serine-threonine kinases that converges in the activation of extracellular signal regulated kinases (ERKs), also known as p42^{mapk} and p44^{mapk}, which ultimately regulate the expression of genes essential for proliferation. Thus, we next investigated how G proteins link cell surface receptors to the activation of ERKs.

Whereas α subunits were thought to be the active components of heterotrimeric G proteins, βγ complexes were believed to simply regulate α activity. However, we have found that βγ subunits of G proteins, not Gα, act in a *ras*-dependent manner to stimulate ERKs (6). Furthermore, G protein-coupled receptors were shown to activate ERK in a βγ-dependent manner. These findings support a novel mechanism for activation of *ras* by βγ complexes, and suggest that signals from G protein-coupled receptors converge at the level of p21^{ras} with the pathway utilized by receptor-tyrosine kinases.

To explore nuclear events involved in growth regulation by G protein-coupled receptors expressed in NIH 3T3 cells, we compared the effect of PDGF and the cholinergic agonist, carbachol, on the expression of mRNA for members of the *jun* and *fos* family of nuclear proto-oncogenes. We found that activation of m1 receptors induces the expression of a distinct set of nuclear transcription factors. In particular, the muscarinic agonist carbachol caused a much greater induction of c-jun mRNA and AP-1 activity (7). These responses did not correlate with the activation of ERKs. Recently, it has been shown that a novel family of kinases structurally related to ERKs, stress

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activated protein kinases (SAPKs) or jun kinases (JNKs), phosphorylate the N-terminal transactivating domain of the c-jun protein thereby increasing its transcriptional activity. In view of our results, we asked whether m1 and PDGF can differentially activate JNKs. We have found that m1 mAChRs can induce a remarkable increase in JNK activity. In contrast, PDGF failed to activate JNK in these cells, although it stimulated ERKs to an extent even greater than that for carbachol (7). These findings demonstrate that stimulation of G protein-coupled receptors can lead to JNK activation, thus diverging at this level with those signaling routes utilized by tyrosine kinase receptors.

The observation that in NIH 3T3 cells JNK can be activated by G protein-coupled receptors but not upon stimulation of receptors of the tyrosine kinase class led us to hypothesize that JNK and ERKs might be differentially regulated, and prompted us to investigate the biochemical route controlling JNK. Using the expression of an epitope-tagged JNK1 (HA-JNK) in COS-7 cells as a model system to explore the mechanism of activation of JNK, we observed that Ras could weakly activate JNK, however, utilizing a signaling pathway distinct from that regulating ERKs (8). In contrast, we found that expression of mutationally activated forms of two members of the Rho-family of small GTP-binding proteins, Rac1 and Cdc42, can initiate an independent kinase cascade leading to JNK activation. Furthermore, we obtained evidence to support that Rac1 and Cdc42 are an integral part of the signaling route linking G protein-coupled receptors as well as receptors for inflammatory cytokines and epidermal growth factor to JNK.

Taken together, these findings strongly suggest that although Ras controls the activity of ERKs, two Rho-related GTPases, Rac1 and Cdc42, regulate the activity of JNK. Thus, the emerging picture from our studies as well as from recently published reports is that distinct small GTP-binding proteins link cell surface receptors with independent signaling pathways which, in turn, lead to the activation of each member of the MAPK superfamily.

Bibliography

1. Gutkind JS, Novotny E, Brann MR, Robbins KC. Proc Natl Acad Sci USA 88, 4703-4707, 1991.
2. Stephens EV, Kalinec G, Brann MR, Gutkind JS. Oncogene 8, 19-26, 1993.
3. Kalinec G, Nazarali AJ, Hermouet S, Xu N, Gutkind JS. Mol Cell Biol 12, 4687-4693, 1992.
4. Xu N, Bradley L, Ambdukar I, Gutkind JS. Proc Natl Acad Sci USA 90, 6741-6745, 1993.
5. Crespo P, Xu N, Daniotti JL, Rapp UR, Gutkind JS. J Biol Chem 269, 21103-9, 1994.
6. Crespo P, Xu N, Simonds WF, Gutkind JS. Nature 369, 418-20, 1994.
7. Coso O, Chiariello M, Kalinec G, Kyriakis J, Woodgett J, Gutkind JS. J Biol Chem 270, 5620-5624, 1995.
8. Coso O, Chiariello M, Yu J.-C, Crespo P, Teramoto, H, Xu N, Miki T, Gutkind JS. Cell 81, 1137-1146, 1995.

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The Rho Family GTPases RhoA, Rac1 And CDC42hs Regulate Transcriptional Activation By SRF

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Many different growth factors and mitogens cause rapid and transient transcriptional activation of cellular "immediate-early" genes such as *c-fos*. A regulatory sequence common to the promoters of many of these genes is the Serum Response Element (SRE). The *c-fos* SRE binds a transcription factor ternary complex comprising Serum Response Factor (SRF) and one of three Ets domain accessory proteins, the Ternary Complex Factors Elk-1, SAP-1 or SAP-2.

The TCFs are direct targets for extracellular signals, and activation of the Ras/Raf/Erk pathway is sufficient for their activation [1]. TCF-binding is required for activation of *c-fos* in response to stimulation by polypeptide growth factors that signal through receptor tyrosine kinases or in response to activation of protein kinase C by phorbol esters [2,3]. However, serum-induced activation of the *c-fos* promoter or SRE-controlled reporter genes is not absolutely dependent on TCF binding [2,3]. TCF-independent activation of the SRE is mediated by SRF, and requires SRF to be targeted to DNA via its own DNA-binding domain [2]. In addition, agents that act through heterotrimeric G protein-coupled receptors, such as the serum mitogen LPA (lysophosphatidic acid), as well as intracellular activation of heterotrimeric G proteins by aluminium fluoride ion (AlF_4^-) also activate this novel SRF-linked signalling pathway [3]. Evidence will be presented which implicates members of the rho family of GTPases in the signalling pathway from heterotrimeric G proteins to the SRE [4]. Activated forms of RhoA, Rac1 and CDC42hs activate transcription via SRF, and act synergistically at the SRE with signals that activate TCF. Functional Rho is required for signalling to SRF by several stimuli including serum, LPA and AlF_4^-, but not by activated CDC42hs or Rac1. Moreover, RhoA can mediate signalling to the SRE by serum, LPA or AlF_4^-. Activation of the SRF-linked signalling pathway does not correlate with activation of the MAP kinases ERK, SAPK/JNK or MPK2/p38. These

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results establish SRF as a nuclear target of a novel Rho-mediated signalling pathway.

1. Treisman, R. (1994) Curr. Opin. Genet. Dev. **4** 96-101.
2. Hill, C. S., Wynne, J. and Treisman, R. (1994).EMBO J. **13**, 5421-5432..
3. Hill, C.S. and Treisman (1995) EMBO J. in press.
4. Hill, C. S., Wynne, J. and Treisman, R. (1995) Cell **81** 1159-1170.

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Rho *p21* and Its Putative Target Molecules

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The small GTP-binding protein Rho shuttles between the inactive GDP-bound form and the active GTP-bound form and works as a molecular switch in such cellular processes as the stimulus-evoked cell adhesion, cytokinesis, the regulation of transcriptional activation, cell cycle progression and cell transformation. The biochemical mechanism of these actions, however, remains unknown. This is apparently due to the lack of identification of the downstream effectors. We have previously demonstrated that several Ser/Thr kinases were activated in a Rho-dependent manner in Swiss 3T3 cells and rat 3Y1 fibroblasts. Using a yeast two hybrid system and a ligand overlay assay with [^{35}S]GTPγS-loaded Rho, we have identified several molecules which specifically bind to the GTP-bound form of Rho. Based on the structure of their Rho binding domain, these molecules can be grouped into two. To one group belong a serine/threonine kinase, PKN and two other molecules containing a region homologous to the regulatory domain of PKN, which we call rhophilin and rhotekin. The other group comprizes a novel serine/threonine protien kinase, p160^{ROCK}, and non-kinase molecule, p183 citron, both of which contain a long coiled-coil structure. Both putative target kinases not only physically associate with GTP-Rho but also are activated by this association both *in vitro* and *in vivo*.

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FIFTH SESSION:
INVOLVEMENT OF G PROTEINS IN CELL FUNCTION (III)

Chairman: Juan Carlos Lacal

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PLA_2 in ras and rac-mediated signalling.

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Growth factor-induced stress fiber formation involves signal transduction through rac and rho proteins, and production of leukotrienes from arachidonic acid. In exploring the relationship between these pathways we found that rac is essential for EGF-induced arachidonic acid production and subsequent generation of leukotrienes, and that rac V12, a constitutively activated mutant of rac, generates leukotrienes in a growth factor-independent manner. Leukotrienes generated by EGF or rac V12 are necessary and sufficient for stress fiber formation. Furthermore, leukotriene-dependent stress fiber formation requires rho proteins. We have therefore identified elements of a pathway from growth factor receptors that includes rac, arachidonic acid production, arachidonic acid metabolism to leukotrienes, and leukotriene-dependent rho activation. This appears to be the major pathway by which rac influences rho-dependent cytoskeleton rearrangements.

Inhibition of rac by the dominant negative mutant rac^{asn17} inhibits EGF-induced calcium influx in Hela Cells and rat 1 cells. This result is in agreement with previous findings that the opening of 10pS calcium channels is mediated by leukotrienes. However, leukotrienes or arachidonic acid alone is not sufficient to induce channel opening.

It has been reported that PI-3K mediates the activation of rac by growth factors. We recently found that one of the downstream element of PI-3K signalling is Akt/PKB. This serine/threonine kinase contains a PH-domain and may be involved in the activation of S6 kinase. The mechanism by which Akt/PKB is activated is still unknown, but it may include binding of lipids (PI-3,4,5-P?) to the PH domain and the induction of autophosphorylation. The relationship between Akt/PKB and rac is currently under investigation.

Rad is a small GTPase first identified in muscle tissue from patients with diabetes type II. In a yeast 2 hybrid screen we found that rad associates with β-tropomyosin. Indeed, both rad and β-tropomyosin colocalize in muscle cells as shown by immunofluorescence. This suggests that rad may be involved in either the formation of sarcomeres or the regulation of contraction.

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Target Molecules for Ras and Rho Small G Proteins

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Ras regulates gene expression at least through the MAP kinase cascade consisting of MAP kinase, MAP kinase kinase (MEK), and MEK kinase (c-Raf-1). Ras is shown to directly interact with c-Raf-1 in a cell-free system and to induce its activation in intact cell systems, but it still remains to be demonstrated that Ras activates c-Raf-1 in a cell-free system. We purified from bovine brain a MEK kinase which activated MEK in a GTPγS-Ki-Ras-dependent manner in a cell-free system. This protein kinase was identified to be B-Raf complexed with 14-3-3 proteins. GTPγS-Ha-Ras also activated B-Raf, but GDP-Ki-Ras or GDP-Ha-Ras was inactive. Lipid-modified Ki-Ras and Ha-Ras were active, but lipid-unmodified form was nearly inactive. By use of this system, we are currently investigating whether Rap1 activates B-Raf or antagonizes the Ras-dependent B-Raf activation.

Rho regulates reorganization of actin filaments and is implicated in at least maintenance of cell morphology, cell motility, and cytokinesis in mammalian cells. Several possible target molecules for Rho have been reported, but it remains to be clarified how Rho regulates these actin-dependent cell functions. We found that Rho is translocated to a membrane ruffling area when cells are stimulated by HGF or TPA. Rho was also translocated to cell-cell adhesion sites when cell-cell adhesion was induced by Ca^{2+}. Rho was also observed at the contractile ring during cytokinesis. The ERM family (Ezrin, Radixin, and Moesin), which directly interacts with actin filaments through its C-terminal region and with a transmembrane protein CD44 through its N-terminal region, is located at these areas. It is possible that Rho regulates reorganization of the ERM family-dependent association of actin filaments with the plasma membrane.

Rho also exists in the yeast *Saccharomyces cerevisiae* and we found that Rho1 is essential for budding process. Histochemical analysis indicates that Rho is translocated to the tip of the bud where actin filaments are associated with the plasma membrane. We isolated six suppressor genes of temperature sensitive RHO1 mutants. Genetic studies indicate that there are regulatory and target genes for RHO1 in these genes.

In this symposium, we will discuss the modes of action of Ras and Rho.

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P120RasGAP and Shc, two SH2-containing proteins that link receptors to Ras

Peter van der Geer, Sandra Wiley, Gerald D. Gish, Venus Ka-Man Lai, Mark Henkemeyer, Tony Pawson

Signal transduction by receptor protein-tyrosine kinase is initiated by ligand binding and receptor dimerization which is followed by autophosphorylation at a number of sites. Autophosphorylation sites provide binding sites for adaptor and signalling molecules that are involved in signal transduction downstream of the activated receptor. Most of these proteins contain SH2 domains that recognize phosphorylated tyrosine residues in the context of carboxy-terminal residues. A large number of adaptor proteins and and enzymes with SH2 domain have been identified, and it is now important to define their precise roles in the signal transduction process.

One of the best characterized signal transduction pathways downstream of growth factor receptors is the Ras-Map kinase cascade. Ras itself is activated by exchange of GTP for GDP, a reaction that is mediated by guanine nucleotide exchange factors such as mSos1, mSos2 and Ras-GRF. Ras is shut off by hydrolysis of GTP, a process that is greatly accelerated by GTPase activating proteins. Several SH2-containing proteins such as p120RasGAP, Shc and the Grb2-Sos complex have been proposed to be involved in regulation of Ras in response to growth factor stimulation.

Three different Ras-GAPs have been identified in mammalian cells: P120-RasGAP, neurofibromin and GAP1^{m}. Of these p120RasGAP contains SH2 domains and is itself phosphorylated on tyrosine following activation of receptor protein-tyrosine kinases. To study the role of p120-RasGAP in Ras regulation we have

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generated fibroblast cell lines from mouse embryo's that lack either p120-RasGAP or neurofibromin at day nine of gestation. In these cells we have analyzed changes in Ras-GTP levels in response to PDGF. Our data show that in Wt cells Ras-GTP levels barely rise in response PDGF. Baseline levels of Ras-GTP are normal in *Gap*$^{-/-}$ cells, but stimulation with PDGF results in an abnormally large increase in Ras-GTP levels. In contrast, *NF1*$^{-/-}$ cells show an increase in the baseline level of Ras-GTP. Our data clearly indicate that in embryonal fibroblasts p120-RasGAP is activated in response to PDGF to moderate the increase in Ras-GTP following growth factor stimulation, neurofibromin appears to regulate baseline levels of Ras-GTP.

Both p120-RasGAP and neurofibromin have been shown to bind to the effector region of Ras, giving rise to much speculation about effector functions for these proteins. We have analyzed MAP kinase activation and increase in DNA synthesis in response to PDGF in our mutant cells lines and have found that neither p120RasGAP nor neurofibromin are essential for these responses. In addition we have found that cellular transformation by either Ras or Src appears independent of 120-RasGAP and neurofibromin.

Shc is an adaptor protein that contains a single SH2 domain at its carboxy-terminus. Shc, is tyrosine phosphorylated in response to a large number of growth factors and cytokines. Upon phosphorylation on tyrosine 317, Shc binds to the SH2 domain of Grb2, and is thereby able to activate the Ras pathway. Shc is therefore implicated as an adaptor protein able to couple normal and oncogenic protein-tyrosine kinases to Ras activation. The function of the Shc amino-terminus has recently been clarified by work in several laboratories. In our laboratory several Shc-like proteins from vertebrates and Drosophola were cloned. Sequence comparison showed that these proteins have high sequence conservation in the amino-terminal 20 residues, in addition to the carboxy-terminus where the SH2 domain is localized.

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To test whether this conserved amino-terminal domain is involved in protein-protein interactions we used GST-fusion proteins and were able to show that the Shc amino-terminal region binds to a number of tyrosine phosphorylated proteins in v-*src*-transformed cells. The Shc amino-terminus also bound directly to ligand-activated EGF and NGF receptors. A P.Tyr-containing peptide modeled after the Shc-binding site in polyoma middle T antigen (Leu-Ser-Leu-Leu-Ser-Asn-Pro-Thr-P.Tyr-Ser-Val-Met-Arg-Ser-Lys) was able to compete efficiently with the activated EGF and NGF receptors for binding to the Shc amino-terminus. This conserved region in the amino-terminus of Shc is now known as phosphotyrosine-binding (PTB) domain.

Binding appears to be specific for phosphorylated tyrosine residues within the sequence Asn-Pro-X-P.Tyr, which is conserved in many Shc binding sites. Both the NGF receptor and the insulin receptor contain an Asn-Pro-X-P.Tyr motif in their juxtamembrane domain. The NGF receptor coprecipitates with Shc but in contrast the insulin receptor does not. To further understand the binding specificity of the Shc PTB domain we have studied the interaction of the Shc PTB domain with these receptors in vitro. To compare the two binding sites we have used phosphotyrosine-containing peptides based on the Asn-Pro-X-P.Tyr sites in these two proteins in competition binding experiments. Our data indicate that hydrophobic amino acid residues five or six residues amino-terminal to the phosphotyrosine (the -5 and -6 positions) are important for high affinity binding. Substitution of the -5 Ser in the insulin receptor peptide, based on the Tyr 960 phosphorylation site, with Ile markedly increased binding of this phosphopeptide to the PTB domain. These results suggest that while the Shc PTB domain recognizes a core sequence of Asn-Pro-X-P.Tyr its binding affinity is modulated by more amino-terminal residues in the ligand, which may therefore contribute to the specificity of

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the PTB-receptor interactions in a fashion analogous to the carboxy-terminal residues in the SH2-binding sites.

In an analysis of residues in the PTB domain required for binding to phosphotyrosine sites, a specific and evolutionarily conserved Arg has been identified (Arg 175 in human Shc) that is uniquely important for ligand binding, and is potentially involved in phosphotyrosine recognition. Binding of Shc to activated receptors through its amino-terminus could leave the carboxy-terminal SH2 domain free for other interactions. In this way Shc may function as an adaptor protein to bring two tyrosine phosphorylated proteins together.

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Regulation of the Ras dependent MAP kinase pathway.

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Ras binds and activates the serine/threonine kinase Raf, and initiates a MAP kinase cascade that leads to cell proliferation. We have developed an in vitro system for Ras-dependent activation of Raf kinase activity. In this system, the specific activity of Raf kinase is increased by about two orders of magnitude, comparable to the activation seen by Ras in vivo. Sites of phosphorylation on Raf activated in vitro are identical to those seen in vivo. Activation depends on efficient binding of Ras to Raf, and is inhibited by neutralizing anti-Ras antibodies. The precise biochemical mechanism by which Ras activates Raf in this system will be discussed. Activation of the kinase cascade leads to phosphorylation of Sos1, and feedback inhibition of the pathway. Phosphorylation of Sos1 by MAP kinase in vitro suggests that this kinase is responsible for de-sensitization of the signalling pathway: the phosphorylated Sos1 protein, bound to Grb2, has a greatly reduced affinity for the EGF-Receptor, which is itself phosphorylated during signalling.

The Ras-dependent kinase cascade promotes cell proliferation. However, activation of another Ras-driven pathway, the Rac and Rho pathway, strongly synergizes with the kinase cascade in promoting uncontrolled cell division in Ras-transformed cells. The roles of Rac and Rho in this process appears to be distinct from the roles of these proteins in promoting morphological changes in cell cytoskeletal structures. Indeed, these effects are suppressed during transformation by mechansims we dont yet understand. The effects of inhibiting Map kinase pathways and Rac and Rho pathways on tumor cell growth in vitro, and other therapeutic strategies for inhibiting Ras-driven pathways in human tumors will be presented.

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p110γ A NOVEL ISOFORM OF PI3-KINASE CATALYTIC SUBUNIT THAT DOES NOT BIND TO p85 BINDS TO GTP-RAS AND IS ACTIVATED *IN VITRO* BY G PROTEIN SUBUNITS

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Phosphatidylinositol 3-kinase (PI3-K) is an heterodimeric enzyme involved in the signal transduction of many growth factors and cytokines in different cell types. It is composed of a 85 kDa regulatory subunit p85 which interacts with other signalling pathway components and a 110 kDa catalytic subunit p110. The enzyme phosphorylates position 3 of the inositol ring in PtdIns, PtdIns 4-P or PtdIns (4,5)-P_2 to create the putative second messengers PtdIns 3-P, PtdIns (3,4)-P_2 and PtdIns (3,4,5)-P_3.So far three isoforms of p85, α , β and γ and two isoforms of p110, α and β, have been described.
Screening a human bone marrow lambda cDNA-library we isolated a clone with a cDNA-insert highly homologous to the C-terminal part of p110 α and β . Further subcloning and sequencing indicated, that it might be a third isoform of p110.
To investigate the tissue distribution two oligopeptides corresponding to an unique and a conserved region of the putative PI3-K were synthesized and rabbit polyclonal sera against them were produced. Using these antibodies the haematopoetic cell lines K562, U937 and Jurkat were investigated by immunoprecipitation and western blotting . In cell lysates and immunoprecipitates a cross reacting protein with a molecular mass of about 105 to 110 kD could be identified . The corresponding mRNA was detected by Northern Blot analysis in all human tissues tested so far . Using baculovirus vectors the new PI3-K was expressed and purified from Sf-9 cells as a GST-fusion protein. The recombinant fusion protein can phosphorylate PI, PIP and PIP_2 at the C-3 position of the inositol ring as determined by TLC and HPLC analysis. Cotransfection studies in Sf-9 cells clearly show, that in contrast to p110α the new PI3-K isoform (p110γ) does not bind to p85α or p85β . This finding prompted us to search for other binding partners and putative regulators of the p110γ . In the course of these experiments we found a strong *in vitro* activation of p110γ by both the βγ-subunits and the GTP-γ-S activated α-subunit of the heterotrimeric G-protein transducin with a half maximal activation at approximately 200 nM $G_t\beta\gamma$ and 200 nM $G_{t\alpha}$ working with liposomes containing PI as a substrate. Moreover, p110γ activity was considerably more sensitive to $G\alpha_{i1}$ ($K_{0.5}$ = 0.5 nM). p110γ is inhibited *in vitro* by nanomolar concentrations of Wortmannin.
p110γ was also shown to bind specifically to GTP-loaded H-Ras as opposed to GDP-H-Ras. p110γ did not bind to the GTP-loaded effector site Ras mutant A38, suggesting that the binding occurs through the effector site of ras. Using baculovirus expressed deletion mutants of p110γ we were able to show that the binding site for Ras lies in the N-terminal half of p110γ, presumably between residues 150 and 451 of the lipid kinase. We were unable to find any *in vitro* activation of p110γ by GTP-ras under different assay conditions nor could we detect any effect of the p110γ binding on the *in vitro* GTPase activity of H-Ras. *In vivo* studies in COS7 cells show, that cotransfection of the constitutively active Ras mutant V12 leads to elevated levels of PI-3,4-P_2 and PIP_3 in p110α -transfected but not in p110γ-transfected cells suggesting different functional interactions.
p110γ is a new PI3-K catalytic subunit isoform with similar *in vitro* substrate specificity, similar sensitivity to Wortmannin and Ras-binding properties as p110α but displaying no binding to p85, which together with the *in vitro* activation by the G Protein subunits suggests a possible role for p110γ in the signal transduction of G-protein mediated events.

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CELL GROWTH INDUCED BY GROWTH FACTORS AND ONCOGENES IN MURINE FIBROBLASTS IS ASSOCIATED TO THE ACTIVATION OF PHOSPHOLIPASE D THROUGH ALTERNATIVE PATHWAYS.

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Phospholipase D (PLD) is activated by a variety of stimuli, including mitogenic stimulation by growth factors and oncogene transformation. Activation of PLD by growth factors requires PKC since depletion of the enzyme by down regulation or direct inhibition by specific drugs, completely abrogates this effect. Transformation by the *ras* and *src* oncogenes is also associated with an increase in basal PLD activity. However, this effect is not dependent upon PKC, suggesting that growth factors and oncogenes may activate PLD by two independent mechanisms. Here, we demonstrate that activation of PLD by phorbol esters is highly enhanced in *ras*-transformed cells, suggesting a synergistic activation of PLD by *ras* oncogenes and PKC. Also, *ras*-transformed cells showed a drastic attenuation of PLD activation induced by growth factors, while receptor function remains unaffected. This attenuation was a result of the specific uncoupling of the PI-PLC pathway, indicating that activation of PLD induced by growth factors is mediated by PI-PLC and PKC activation. Neither the synergism with PKC activation nor the attenuation of the PLD response to growth factors in *ras*-transformed cells was a general consequence of cell transformation, since cells transformed by other oncogenes showed a normal response to either treatment. Furthermore, attenuation of PLD activation by PDGF was also observed in several oncogene-transformed cells, as a consequence of the uncoupling of the PI-PLC pathway. These results support the existence of at least two alternative signalling routes for the activation of PLD, one mediated by the PI-PLC/DAG/PKC pathway and a second one mediated by several oncogenes including *ras* , independent of the PKC pathway, which synergises with the PI-PLC and PKC-dependent pathway.

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POSTERS

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FUNCTIONAL ACTIVITY OF G_S PROTEIN DURING EXOCRINE PANCREATIC PROLIFERATION IN THE RAT

María del Carmen Boyano-Adánez, Itziar Álvaro-Alonso, Rosa María Izquierdo-Claros, Lilian Puebla, Gema Muñoz-Acedo, Eulalia Rodríguez-Martín and Eduardo Arilla. Departamento de Bioquímica y Biología Molecular, Universidad de Alcalá, E-28871 Alcalá de Henares, Madrid, Spain

Adenylate cyclase activity in pancreatic acinar cell membranes was determined in rats that had undergone a treatment with pentagastrin (250 μg/kg, intraperitoneal three times daily) for 1 week or that had undergone small bowel resection (90%) and were sacrified at 2 weeks, 1 month and 6 months after intervention. Both treatments are potent stimulators of pancreatic acinar cell proliferation. Adenylate cyclase activity was similar under basal conditions and after the diterpene forskolin stimulation in pancreatic acinar membranes from all groups studied. The ability of low concentrations of the stable GTP analogue, 5'-guanylylimidodiphosphate (Gpp[NH]p) to inhibit forskolin- stimulated adenylate cyclase activity was decreased in pancreatic acinar membranes from enterectomized rats at 2 weeks and 1 month after the operation and returned to control values at 6 months after enterectomy. Stimulation of adenylate cyclase by high concentration of Gpp[NH]p or by secretin (10^{-8} M) was higher in both pancreatic hyperplasia conditions as compared with control animals. These findings suggest that the coupling efficiency of the G_s protein to adenylate cyclase from pancreatic acinar membranes is enhanced without any alterations in the catalytic activity of the enzyme during pancreatic proliferation. In addition, it is possible that the highly regulated pancreatic acinar adenylate cyclase activity may be necessary to regulate pancreatic acinar cell proliferation.

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$G\alpha_{12}$ and $G\alpha_{13}$ Stimulate Rho-Dependent Stress Fiber Formation and Focal Adhesion Assembly

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Rho, a member of the Ras superfamily of GTP binding proteins, regulates actin polymerization resulting in the formation of stress fibers and the assembly of focal adhesions. In Swiss 3T3 cells heterotrimeric G protein-coupled receptors for lysophosphatidic acid (LPA) and gastrin releasing peptide (GRP) stimulate Rho-dependent stress fiber and focal adhesion formation. The specific heterotrimeric G protein subunits mediating Rho-dependent stress fiber and focal adhesion formation have not been previously defined. We have microinjected GTPase-deficient, constitutively activated G protein α subunits and mixtures of β and γ subunits into Swiss 3T3 cells. Measurement of actin polymerization and focal adhesion formation indicated that GTPase-deficient α_{12} and α_{13}, but not the activated forms of α_{i2} or α_q stimulated stress fiber formation and focal adhesion assembly. Combinations of β and γ subunits were unable to stimulate stress fiber or focal adhesion formation. $G\alpha_{12}$ and α_{13}-mediated stress fiber and focal adhesion assembly was inhibited by botulinum C3 exoenzyme, which ADP-ribosylates and inactivates Rho, indicating that α_{12} and α_{13} but not other G protein α subunits or $\beta\gamma$ complexes regulate Rho dependent responses. The results define the integration of G_{12} and G_{13} with the regulation of the actin cytoskeleton.

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THE ACTIVITY OF THE JNK/SAPK SIGNALING PATHWAY IS REGULATED BY THE SMALL GTP-BINDING PROTEINS RAC AND CDC42.

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Mitogen-activated protein kinases (MAPKs) are protein serine/threonine kinases that are rapidly activated upon stimulation of a variety of cell surface receptors. c-Jun amino-terminal kinases or Stress Activated Protein Kinases (JNKs/SAPKs) are a novel class of mammalian enzymes closely related to MAPKs, however, they are independently regulated by a variety of environmental stimuli. Although molecules linking growth factor receptors to MAPKs have been recently identified, little is known about the pathways that control JNK activation. Here, we show that in COS-7 cells, activated Ras effectively stimulates MAPK but induces JNK activity only to a poor extent. In contrast, mutationally activated Rac1 and Cdc42 GTPases potently activate JNK without affecting MAPK, and oncogenic guanine-nucleotide exchange factors (GEFs) for these Rho-like proteins, like Dbl and Ost, selectively stimulate JNK activity. Furthermore, expression of inhibitory molecules for Rho-related GTPases and dominant-negative mutants of Rac1 and Cdc42 block JNK activation by oncogenic exchange factors, or when induced by signals originated at cell membrane receptors, as the ones for inflammatory cytokines, growth factors or G protein coupled receptors. Taken together, these findings strongly support a critical role for Rac1 and Cdc42 in controlling the JNK signaling pathway. The participation of these small GTP binding proteins in biochemical routes that connect cell surface receptors to the nucleus will be discussed.

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INDUCTION OF APOPTOSIS BY rho IN NIH 3T3 CELLS REQUIRES TWO COMPLEMENTARY SIGNALS. CERAMIDES FUNCTION AS A PROGRESSION FACTOR FOR APOPTOSIS.

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We have previously reported that rho genes, members of the ras superfamily, are tumorigenic when overexpressed in NIH-3T3 cells. As other known oncogenes, they also induce apoptosis after serum deprivation but not in the presence of growth factors. In the present study, we provide evidence that overexpression of the Aplysia Rho protein in NIH 3T3 cells induces the generation of phosphatidylcholine (PC)-derived second messengers as a result of activation of a PC-specific phospholipase D (PC-PLD) as previously reported for ras-transformed cells. In contrast, removal of serum in the Rho transfectants, but not in normal NIH 3T3 cells or cells transformed by the ras oncogene, induced the production of ceramides as a result of activation of an sphingomyelinase (SMase). Furthermore, the rho-expressing cells underwent apoptosis in the presence of serum when exogenous ceramides were added, and this process was accelerated if cells were treated with exogenous SMase. Thus, Rho proteins act as an initiation signal that is necessary but not sufficient for the induction of apoptosis in NIH 3T3 cells. We propose here that induction of apoptosis in NIH 3T3 cells requires two complementary signals: an initiation signal generated even in the presence of serum which "primes" the cells, making them sensitive to a progression signal, triggered by serum removal, which we have identified as generation of ceramides.

Jiménez B., Arends M., Esteve P., Perona R., Sanchez R., Ramón y Cajal S,., Wyllie A. and Lacal J.C. (1995). Induction of apoptosis in NIH3T3 cells after serum deprivation by overexpression of rho-p21, a GTPase protein of the ras superfamily. Oncogene 10, 811-816.

Perona R., Esteve P., Jimenez B., Ballestero R.P., and Lacal J.C. (1993). Tumorigenic activity of *rho* genes from *Aplysia californica*. Oncogene 8, 1285-1292.

Esteve P., del Peso L, and Lacal J.C. (1995). Induction of apoptosis by rho in NIH3T3 cells requires two complementary signals. Ceramides function as a progression factor of apoptosis. Oncogene. In press.

Instituto Juan March (Madrid)

ROLE OF G PROTEINS IN PVG RAT NATURAL KILLER (NK) CELL MEDIATED TUMOR OR ALLOGENEIC CELL KILLING.

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G proteins play a major role in mediating human NK cell cytotoxicity against tumor cells (JBC 269:6796; 1994). Here, we observed that pretreatment of PVG rat NK cells with pertussis toxin or cholera toxin reversed their killing of all tumor and allogeneic targets, but has no influence on their inability to kill syngeneic target cells. Immunoblot analysis showed that membranes of PVG NK cells possess a 39 Kd G_{i1}, a 39 Kd G_o, a 45-47 Kd G_s and a 45 kD G_z proteins. However, these membranes did not express G_{i2} or G_{i3}. Permeabilization of PVG NK cells with streptolysin O and examining them under fluorescence microscope confirmed these results. Introduction of anti-G protein antibodies (1:10 dilution) inside permeabilized cells showed that anti-G_o and anti-G_z antibodies inhibit their ability to kill tumor and allogeneic target cells (PVG anti-PVG.1U, or PVG.1U anti-PVG). However, this treatment did not reverse their inability to kill syngeneic target cells.

Conclusion: Certain G proteins play highly important roles in transducing signals activating rat NK cell cytotoxicity against allogeneic or tumor target cells but apparently play no role in inhibition of cytotoxicity against syngeneic target cells, suggesting that NK cell recognition of non-self molecules utilizes different signaling pathway (s) than the putative recognition of self-MHC molecules.

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OVEREXPRESSION OF PKCζ DOES NOT INDUCE CELL TRANSFORMATION NOR TUMORIGENICITY AND DOES NOT ALTER NFkB ACTIVITY.

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Signal transduction is the major mechanism by which cells communicate among themselves through extracellular stimuli. Among the different structural components involved in signal transduction, protein kinases are one of the key elements in the process. Protein kinase C is a multimember family of kinases which has been involved in the regulation of diverse cellular functions. Regulation of cell growth in fibroblasts has been reported to be one of such functions. In particular the PKCζ isoenzyme has been postulated to be transforming to NIH3T3 cells (Berra *et al.*, 1993) and to serve as an effector for Ras proteins through the regulation of the NFκB transcription factor (Dominguez *et al.*, 1993) and direct interaction (Díaz-Meco *et al.*, 1994). We have investigated the effects of overexpressing the mouse wild-type PKCζ in NIH3T3 cells. When compared to the parental NIH3T3 cells, we have found 1) no significant effect on cell morphology; 2) no difference in growth properties in the absence of serum or in the presence of individual growth factors such as insulin, phorbol esters or PDGF; 3) no growth in soft agar nor tumorigenic activity in nude mice. In addition cells stably overexpressing the PKCζ kinase did not interfere or amplify the induction of NFκB activity by tumor necrosis factor alfa (TNF-α) nor altered NFκB activity in transient expression of cells treated with TNF-α. Thus, mammalian PKCζ is most likely not directly involved in the regulation of cell proliferation in fibroblasts nor affects directly or indirectly the activation of NFκB.

1. Berra, E., et al. (1993). *Cell,* 74, 555-563.
2. Díaz-Meco, M.T., et al., (1994). *J.Biol.Chem.*, 269, 31706-31170.
3. Dominguez, I. et al. (1993). *Mol.Cell.Biol.*, 13, 1290-1295.

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CYTOSOLIC PHOSPHOLIPASE A_2 IS COUPLED TO MUSCARINIC RECEPTORS IN THE HUMAN ASTROCYTOMA CELL LINE 1321N1

Study of the mechanisms involved in this coupling

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The cholinergic agonist carbachol induced the release of arachidonic acid in the 1321N1 astrocytoma cell line, and this was blocked by atropine suggesting the involvement of muscarinic receptors. To assess the mechanisms of signaling involved in the response to carbachol, a set of compounds characterized by eliciting responses through different mechanisms was tested. A combination of PMA and thapsigargin, an inhibitor of endomembrane Ca^{2+}-ATPase that induces a prolonged elevation of cytosolic Ca^{2+} concentration, induced an optimal response, suggesting at first glance that both PKC and Ca^{2+}-mobilization were involved in the response. This was consistent with the observation that carbachol elicited Ca^{2+}-mobilization, and enhanced phosphorylation of cytosolic phospholipase A_2 ($cPLA_2$) as measured by a decrease in electrophoretic mobility. However, the release of arachidonate induced by carbachol was unaltered in media containing reduced concentrations of Ca^{2+} or in the presence of neomycin, a potent inhibitor of phospholipase C which blocks phosphoinositide turnover and Ca^{2+}-mobilization. The effect of carbachol was enhanced in cells treated with AlF_4^-, and GTPγS added to the cell-free homogenate induced both [^{3}H]arachidonate release and $cPLA_2$ translocation to the cell membrane fraction in the absence of Ca^{2+}; all of these events suggesting the existence of an alternative mechanism of signaling dependent on G-proteins and independent of Ca^{2+}-mobilization. From the combination of experiments utilizing pharmacological, biochemical, molecular and immunological tools the involvement of $cPLA_2$ was ascertained. In summary, these data indicate the existence in the astrocytoma cell line 1321N1 of a pathway involving the $cPLA_2$ which couples the release of arachidonate to the occupancy of receptors for a neurotransmitter.

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Stimulation of cloned human 5-$HT_{1D\beta}$ receptor sites in stably transfected C6-glial cells promotes cell growth Petrus J. Pauwels, Thierry Wurch, Marie-Claude Amoureux, Christiane Palmier and Francis C. Colpaert, Laboratory of Cellular and Molecular Neurobiology, Centre de Recherche Pierre Fabre, 81106 Castres Cedex, France

The involvement of 5-$HT_{1D\beta}$ receptor sites was investigated in the growth of rat C6-glial cells permanently transfected with a gene encoding a human serotonin $(5\text{-HT})_{1D\beta}$ receptor. The 5-HT receptor identity of control and transfected C6-glial/5-$HT_{1D\beta}$ cells was determined by reverse transcription-polymerase chain reaction (RT-PCR) using primers specific for rat 5-HT_{1B}, human 5-$HT_{1D\alpha}$, human 5-$HT_{1D\beta}$ and rat 5-HT_{2A} receptor genes. Constitutive mRNA for 5-HT_{2A} receptors was present in control and transfected C6-glial/5-$HT_{1D\beta}$ cells, whereas mRNA for 5-$HT_{1D\beta}$ receptor sites was only present in the transfected C6-glial/5-$HT_{1D\beta}$ cell line. 5-HT inhibited forskolin-stimulated cAMP formation and promoted cell growth in contrast to the absence of any measurable effect in pcDNA3 plasmid-transfected non-transfected C6-glial cells. The 5-HT effects could be mimicked by sumatriptan (EC_{50}: 44 to 76 nM), and were totally and partially blocked by methiothepin (IC_{50} : 9 nM) and GR 127,935 (2'-methyl-4'-(5-methyl-[1,2,4] oxadiazol-3-yl)-biphenyl-4-carboxylic acid [4-methoxy-3-(4-methyl-piperazin-1-yl)-phenyl]-amide, IC_{50} : 97 pM), respectively. No effect on cell growth was measured with the 5-HT_2 receptor agonist DOI (1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane, 10 μM) suggesting that 5-HT_{2A} receptors are not involved in the 5-HT-stimulated C6-glial/5-$HT_{1D\beta}$ cell growth. Dibutyryl-cAMP (0.3 mM) treated cultures did not show sumatriptan-promoted cell growth indicating an inhibitory role for cAMP in the cell growth mediated by 5-$HT_{1D\beta}$ receptor sites. In conclusion, 5-HT promotes cell proliferation in transfected C6-glial/5-$HT_{1D\beta}$ cells by stimulation of 5-$HT_{1D\beta}$ receptor sites. These receptor sites may be a new target to modulate the growth of tumor cells.

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Isolation and characterisation of *CLT1*, a *Candida albicans* gene which codes a small GTPase, closely related to *Saccharomyces cerevisiae TEM1* gene

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The incidence of fungal infections is increasing among industrialised countries[1]. In contrast, the therapeutic arsenal has not increased significantly in the last years, and so, there is a general belief in the importance of identification of novel potential targets as a major force towards the designing of novel antifungals. *Candida albicans* is a dimorphic pathogenic fungus which is a normal comensal of human being, normally located at the gastrointestinal tract, oral cavity and vagina. Under certain circumstances which are (not only but mainly) related with the state of immunocompetence of the individual, this microorganism is able to gain access to other tissues and establish an infection which, if systemic, is often life threatening. Since genetic manipulation of *Candida albicans* is hampered by the fact of its diploidy and lack of sexual cycle, we have used *Saccharomyces cerevisiae* as a genetic host for the identification and analysis of potentia novel antifungals.

The cell wall of *Candida albicans* is, due to its characteristics and location in the cell, one of the most promising targets for the development of novel antifungals. We have therefore used *Saccharomyces cerevisiae* conditional thermosensitive lytic mutants for the screening of a *Candida albicans* genomic gene library. *Saccharomyces cerevisiae lyt1-1* is a thermosensitive mutant[2,3], unable to grow at 37°C and sporulate, which has been the subject of previous work in our Department. We have used this mutant as genetic host, cloning in this way a region of DNA able to complement the thermosensitiveness but not the meiotic defect. The analysis of a 4 kbp region has revealed an ORF of 675 pb responsible for this complementation which has been named *CLT1* (for Candida LYT1 suppressor). The predicted protein coded by this gene (Clt1p) has an elevated degree of similarity to the members of small eukaryotic GTPases[4], although it has a slightly reduced molecular size Clt1p is 48,2% similar to the product of *TEM1* de *S. cerevisiae*[5], and 20%, 18% and 5,2% similar to the product of *S. cerevisiae SEC4*, *YPT1* and *RHO4* genes, although it is unable to complement *sec4*, *ypt1* and *rho4* mutations. It has been suggeste that Tem1p would be a representant of novel branch of small eukaryotic GTPases. This gene, a multicopy suppressor of *lyt1-1* mutant seems to be essential in *Candida albicans* since we have been unable to delete both alleles of the gene. We are currently in the process of analysing its ability to complement *Saccharomyces cerevisiae tem1-3* and *tem1Δ* mutants and constructing thermosensitive alleles to analyse its role in cell divisior

1. **Fox, J.L.** 1993. Fungal infection rates are increasing. *ASM News* 59: 515-518.
2. **Cabib, E. y Durán, A.** 1975. Simple and sensitive procedure for screening yeast mutants that lyse non-permissive temperatures. *J. Bacteriol.* 124: 1604-1606.
3. **Molero, G., Yuste Rojas, M., Montesi, A., Vazquez, A., Nombela, C. y Sanchez, M.** 1993. A *cdc*-li autolytic *Saccharomyces cerevisiae* mutant altered in budding site selection is complemented by *SPO* a sporulation gene. *J. Bacteriol.* 175: 6562-6570.
4. **Bourne, H.R., Sanders, D.A. y McCormick, F.** 1991. The GTPase superfamily: conserved structure a molecular mechanism. *Nature* 349: 117-127.
5. **Shirayama, M., Matsui, Y. y Toh, E., A.** 1994. The yeast *TEM1* gene, which encodes a GTP-bind protein, is involved in termination of M phase. *Mol. Cell. Biol.* 14: 7476-7482.

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SH3 domain interactions and dynamin assembly

Dynamin is a 100 kD GTPase necessary for endocytosis. The proline rich C-terminus is necessary for self-assembly as well as for interaction with SH3 domains. Separate C-terminal proline-motifs represent distinct binding sites for different SH3 domain proteins, and interaction with SH3 domain proteins (such as PLCγ-1) may provide a link between dynamin and activated receptor tyrosine kinases (Scaife et al, EMBO J. 1994). We have subsequently investigated the role of SH3 domain interactions on dynamin assembly. While dynamin binds to the SH3 domain of a range of signal transduction proteins, we found that a subset of SH3 domains greatly promote dynamin assembly. Stimulation of dynamin *in vitro* assembly occurs at very substoichiometric levels of SH3 domain. Our results therefore indicate that dynamin SH3 interactions may represent a critical step in the assembly of the endocytic machinery.

Instituto Juan March (Madrid)

A Small GTP-binding Protein, Rho, Associates with the Platelet-Derived Growth Factor Type-ß Receptor

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Ligand binding to the platelet-derived growth factor (PDGF) receptor initiates a complex and diverging cascade of signaling pathways. GTP-binding proteins (G-proteins) frequently link cell surface receptors to intracellular signaling pathways, but no close associations of the PDGF receptor and any small G-proteins, nor any such associations activated by ligand binding to the receptor have been previously reported. We demonstrate that a small GTP-binding protein binds specifically to the murine and human PDGF type-β receptor. In response to PDGF-BB stimulation, there is an increase in the amount of labeled small G-protein associated with the PDGF type-ß receptor. The GTP-binding protein did not undergo ligand-induced association with a mutant receptor protein which was unable to bind ATP. Proteolytic cleavage analysis together with two-dimensional separation techniques, identified the small G-protein specifically associating with the PDGF type-β receptor after ligand binding as a member of the Rho family. This was confirmed by demonstration that the small G-protein co-immunoprecipitated by the anti-PDGF receptor antibody was a substrate for the ADP-ribosyltransferase C3 exoenzyme. Thus, the PDGF type-β receptor may form a complex with one o more small G-proteins upon binding PDGF-BB, and the Rho small G-protein is likely to be an important component of the proteins making up the multimeric signaling complex of the PDGF type-β receptor.

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List of Invited Speakers

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Workshop on

G-PROTEINS: STRUCTURAL FEATURES AND THEIR INVOLVEMENT IN THE REGULATION OF CELL GROWTH

List of Invited Speakers

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G-PROTEINS: STRUCTURAL FEATURES AND THEIR INVOLVEMENT IN THE REGULATION OF CELL GROWTH

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