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267 Workshop on Role of Glycosyl-Phosphatidylinositol in Cell Signalling

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I N D E X

P R O G R A M M E

ROLE OF GLYCOSYL-PHOSPHATIDYLINOSITOL IN CELL SIGNALLING

Monday, October 21st, 1991

Welcome & Introduction: J.M. MATO

1st Session - Chairman: J.M. MATO

Joe Larner - Chiro-inositol: a new player in diabetic insulin resistance.

Alan R. Saltiel - Regulatory properties of inositol glycans.

Martin G. Low - GPI-specific phospholipase D: a plasma enzyme that degrades the membrane anchor of cell surface proteins.

2nd Session - Chairman: J. LARNER

Guillermo Romero - Insulin and IGF-1 induce the generation of inositolglycans in the extracellular medium.

Robert V. Farese - Phospholipid metabolism and protein kinase C activation in insulin action.

Claude Jacquemin - Biological effects of glycosyl inositol-phosphate on adipocytes and thyrocytes: Role of pertussis toxin sensitive G proteins.

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Isabel Varela-Nieto - Role of the glycosyl-phosphatidylinositol/ inositol phosphoglycan signalling system in the regulation of gene expression and cell proliferation.

Glen N. Gaulton - Regulation of T Lymphocyte growth and function by GPI.

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Peter Strålfors - Inositol-glycan and diacylglycerol: dual second messengers of insulin ?

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Enrique Rodríguez-Boulan - GPI acts as an apical targeting signal in polarized epithelial cells.

Geneviève Rougon - Expression and release of glycosyl-phosphatidylinositol anchored molecules during neuronal differentiation.

Manuel Martín-Lomas - Inositol glycans as insulin mimetics: structural studies and new synthetic approaches.

Concluding remarks: J. LARNER

INTRODUCTION

ROLE OF GLYCOSYL-PHOSPHATIDYLINOSITOL IN CELL SIGNALLING: INTRODUCTION

José M. Mato. Instituto de Investigaciones Biomédicas, CSIC, Arturo Duperier 4, 28029-Madrid

Glycosyl phosphatidylinositols are found in a diversity of organisms from bacteria and yeast to mammalian cells. In 1986, Saltiel and Cuatrecasas first reported that the addition of insulin to BC3H1 myocytes produced a fast and transient hydrolysis of a glycosyl phosphatidylinositol (GPI) with generation of diacylglycerol and the polar headgroup of the lipid, an inositol phosphoglycan named IPG. Since then, several laboratories have reported a similar effect of insulin on GPI hydrolysis using hepatoma cells, hepatocytes, adipocytes, lymphocytes and CHO cells. The detailed structure of IPG is not known but it has been reported to contain **myo-** and **chiro-**inositol, glucosamine, galactosamine, galactose and mannose. A variety of results indicate a strong structural similarity between IPG and the glycan moiety of glycosyl phosphatidylinositol molecules that serve as protein anchors. Thus, an antibody risen against the glycan moiety of **T. brucei** variant surface glycoprotein (VSG) has been found to cross react with IPG obtained from both rat liver and chick embryo GPI. This antibody is specific and did not react with inositol, inositol phosphate, glucosamine, galactose or mannose.

In addition to insulin, other growth factors and hormones have been found to stimulate GPI hydrolysis in target cells. The receptors for these ligands fall into two categories: the receptor protein-tyrosine kinase family, formed by the insulin, IGF-I, EGF, NGF and IL-2 receptors, and the group of receptors with seven transmembrane domains, formed by the ACTH and TSH receptors. These results suggest the existence of two mechanisms leading to GPI hydrolysis, one dependent and the other one independent of protein-tyrosine kinase activity.

Hydrolysis of GPI in response to insulin is reduced in CHO cells bearing protein-tyrosine kinase-deficient insulin receptors. Cells carrying normal human receptors hydrolysed up to 70% of their GPI within 2 min of the addition of 0.1 nM insulin, whereas parental cells and cells expressing the mutant receptor hydrolysed only 20-30% in response to 100 nM insulin. These results indicate that the receptor protein-tyrosine kinase is necessary to transduce efficiently the effect of insulin on GPI hydrolysis. Insulin-stimulated GPI hydrolysis can be decreased in myocytes by treatment with cholera toxin. Thus, it is possible that G proteins are an intermediate in the signal cascade from receptor to phospholipase activation. Insulin binds to the α subunit of the insulin receptor which stimulates the protein-tyrosine kinase activity of the β subunit. The mechanism by which the insulin receptor protein-tyrosine kinase and GPI hydrolysis are linked is not known.

NGF-induced GPI hydrolysis has been observed in PC12 and chick embryo chocleovestibular ganglion (CVG). The high affinity NGF

receptor appears to be a heterodimer composed by the protein-tyrosine kinase **trk** gene product, a 140K protein, and the low-affinity NGF receptor (NGFR), a 75K protein. A model has been proposed where NGF exists as a non covalent dimer that binds both to the 75K and 140K proteins. In PC12, NGF binding induces autophosphorylation by the **trk**-encoded protein-tyrosine kinase suggesting that, as in the case of insulin, the receptor protein-tyrosine kinase is required for activation of GPI hydrolysis. There are two well characterized members of the **trk** family, **trk** and **trk**B, as well as several uncharacterized members, which are all 130-140K proteins expressed in distinct regions of the nervous system. NGF is also one of at least three related neurotrophic factors; NGF, brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3). BDNF and NT-3, but not NGF, are ligands for the **trk**B protein-tyrosine kinase receptor. Binding to the **trk**B receptor by BDNF and NT-3 is of low affinity and it has been proposed that, by analogy to NGF, high affinity binding sites for BDNF and NT-3 might be obtained by the interaction of **trk**B with NGFR or NGFR-like molecules. While the components of the signal-transducing pathway of BDNF or NT-3 have not yet been identified, by similarity with NGF, these two neurotrophic factors may induce GPI hydrolysis in specific subpopulations of neurons.

Another example of a multisubunit receptor associated with a protein tyrosine kinase is the IL-2 receptor. The IL-2 receptor appears to be composed by at least two distinct polypeptides, the IL-2 receptor α, a 55K protein, and the IL-2 receptor ß, a 70-75K protein that belongs to the cytokine receptor family. Similarly to the situation with the NGF receptor, together the two receptor subunits form the high affinity IL-2 receptor. The IL-2 receptor subunit interacts directly with the lymphocyte specific protein-tyrosine kinase $p56^{lck}$, a member of the srk-family kinases. As a result of this interaction, in the presence of IL-2 $p56^{lck}$ protein tyrosine kinase activity is stimulated and the IL-2 receptor ß subunit becomes phosphorylated. Studies of IL-2- and IL-4-induced responses in B lymphocytes indicate that exposure to IL-2 is rapidly followed by GPI hydrolysis and proliferation of the lymphoma cells, and that both responses are blocked when cells are exposed to IL-4 for 5 min, which by itself has no effect on GPI hydrolysis. This rapid antagonism does not seem to be mediated by down-regulation of IL-2 receptors, and a model has been proposed where IL-4 exerts its antagonistic action by preventing the association of the IL-2 receptor with the protein-tyrosine kinase. In this model the phosphotyrosine containing receptor, may interact with a GPI specific phospholipase or with other physiologic regulators leading to phospholipase activation. In addition, these results suggest that other members of the cytokine receptor family may behave similarly to the IL-2 receptor and stimulate GPI hydrolysis.

The discovery that the insulin receptor activates GPI hydrolysis prompted work on the cellular responses to IPG. This molecule, added to intact cells, inhibits lipolysis and stimulates lipogenesis and glucose consumption in adipocytes, reduces glycogen phosphorylase and cAMP levels, and stimulates

pyruvate kinase and glycogen synthesis in hepatocytes. In fibroblasts IPG stimulates lactate and fructose 2,6-bisphosphate production. Furthermore, this molecule has been found to elicit the phosphorylation and dephosphorylation of some of the same cellular proteins as insulin (i.e. ATP citrate lyase, glycogen phosphorylase, hormone sensitive lipase). This suggests that cAMP-dependent and independent protein kinases and phosphoprotein phosphatases might be the intracellular targets of IPG. At this respect, in broken cell preparations or with purified enzymes IPG stimulates cAMP phosphodiesterase, pyruvate dehydrogenase and casein kinase II and inhibits adenylate cyclase and cAMP dependent protein kinase.

IPG has no effect on glucose transport, but diacylglycerol has been shown to act as an activator of this process in adipocytes when applied outside the cell, and addition of phospholipase C to intact adipocytes also stimulated glucose transport. Thus it is possible that insulin generates two types of biochemical signals: IPG and diacylglycerol. GPI hydrolysis is not the only source of diacylglycerol in response to insulin and in myocytes this hormone has been shown to stimulate the **de novo** synthesis of diacylglycerol. Sphingosine, a reversible inhibitor of protein kinase C which acts competitively with respect to activators such as phorbol esters and diacylglycerol, has been shown to inhibit insulin-stimulated glucose transport in 3T3-L1 fibroblasts and adipocytes, which is consistent with a role for diacylglycerol and protein kinase C in the activation of glucose transport by this hormone.

The ability of IPG to regulate the expression of specific genes has also been investigated. Incubation of hepatocytes with IPG produced a 5-fold decrease in phosphoenolpyruvate carboxykinase (PEPCK) mRNA levels, which had been previously increased about 10-fold by incubation with 0.1 mM 8-Br-cAMP. IPG reduction of PEPCK mRNA is primarily due to a decrease in the rate of transcription of the gene as judged by nuclear run-on transcription experiments performed in rat hepatoma H4IIE cells. In hepatocytes isolated from diabetic rats, treatment with IPG caused a 4-fold induction in the expression of α2-microglobulin mRNA, concomitantly with a 2.5-fold decrease in the level of PEPCK mRNA. Similarly, IPG has also been found to stimulate p33 mRNA accumulation. These data indicate that IPG has both positive and negative effects on the regulation of gene expression.

Both insulin and NGF have been found to behave as growth factors in otic vesicles cultured **in vitro**. The otic vesicle is a transient embryonic structure occurring during the early development of the inner ear. Insulin potentiates the proliferative effects of other growth factors on the epithelium of quiescent otic vesicles and NGF behaves as a potent mitogen of the CVG. IPG, like insulin, potentiates the mitogenic effect of bombesin on the otic vesicle epithelium and, like NGF, is a potent mitogen of the ganglionic cell population. Moreover, the IPG- and NGF-induced proliferative effect on CVG are blocked by anti-IPG antibodies. Similarly, IPG has also been found to potentiate the mitogenic effect of IL-2 in T-lymphocytes and to

stimulate proliferation of NIH 3T3 fibroblasts. These findings point to IPG as an important modulator of cell proliferation.

Anti-IPG antibodies, generated against the glycan moiety of **T. brucei**, block both the NGF-induced proliferative effect on CVG and the insulin-induced stimulation of pyruvate dehydrogenase (PDH) in intact BC3H1 myocytes. Moreover these antibodies block the **in vitro** effects of IPG on mitochondrial PDH and cAMP-dependent protein kinase as well as the proliferative effect of IPG on CVG. When added to intact cells in the presence of insulin, these antibodies induce the accumulation of IPG in the extracellular medium and had no effect on insulin-stimulated glucose uptake and diacylglycerol production. These results indicate that IPG is generated outside the cell in response to insulin or NGF. This hypothesis is further supported by the finding that the majority of the GPI is present on the cell surface of insulin-sensitive cells and by the observation that in TSH treated pig thyroid cells IPG accumulates in the incubation medium. Moreover, the specific uptake of IPG by intact rat hepatocytes has also been recently demonstrated. These and other related observations support a model where IPG originates from GPI molecules present on the cell surface. As a result of this, IPG is released to the incubation medium to be then taken up by a specific uptake system.

Impaired insulin-dependent GPI hydrolysis has been observed in hepatocytes from glucocorticoid-treated rats (a model of type II diabetes) and in adipocytes from streptozotocin-induced diabetic rats (a model of type I diabetes). Moreover, IPG was undetectable in muscle biopsies from patients with non-insulin-dependent diabetes mellitus (NIDDM) and the urinary excretion of chiro-inositol (a component of IPG) in NIDDM was lower than in control subjects. These results seem to indicate a deficient metabolism of GPI in diabetes and open the possibility that IPG and/or IPG-analogs may be of potential interest in the treatment of this disease.

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1st SESSION

CHIRO-INOSITOL: A NEW PLAYER IN DIABETIC INSULIN RESISTANCE

J. Larner, G. Romero, I. Asplin, G. Galasko, L.C. Huang, C. Zhang
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Two phospho inositol glycan insulin mediators have been separated from liver and muscle in our laboratory by anion exchange chromatography. The pH 2.0 eluted mediator which contains D-chiro-inositol and galactosamine stimulates pyruvate dehydrogenase (PDH) phosphatase in a manner kinetically like insulin. It also stimulates glycogen synthase phosphatase. The pH 1.3 eluted mediator which contains myo-inositol and glucosamine inhibits CAMP-dependent protein kinase and adenylate cyclase. Since insulin resistance in type 2 diabetes is principally post-receptor and mainly in non-oxidative glucose metabolism (glycogen synthesis) we examined the role of the two mediators by measuring (1) myo- and chiro-inositol in 24 hour urine samples, (2) bioactivity and myo- and chiro-inositol in mediator samples from urine, hemodialysate and muscle of control and diabetic subjects. Urine inositol analysis indicates that about 80% of type 2 diabetic subjects have a marked decrease or absence of chiro-inositol with 70% of type 1 diabetic subjects similarly affected. Analysis of mediator activity and inositol content in muscle, hemodialysate and urine samples confirm the selective decrease in pH 2.0 mediator and chiro-inositol content in type 2 diabetic subjects. This defect appears related to insulin resistance and may have a familial or genetic component. A similar defect is observed in the Pima Indians and in a collection of prediabetic and diabetic monkeys.

REGULATORY PROPERTIES OF INOSITOL GLYCANS, A.R. SALTIEL, Parke-Davis Pharmaceutical Research Division, Ann Arbor, MI 48105, USA

It has recently been discovered that over forty proteins of diverse origin and function are anchored to the plasma membrane by covalent attachment to glycosyl-phosphoinositides. In these novel structures, proteins are linked via an amide bond to ethanolamine, which is coupled to an oligosaccharide glycosidically linked to phosphatidylinositol (PI). We have found a structurally homologous form of this glycolipid that apparently is not attached to protein. This molecule is hydrolyzed by a specific phospholipase C, giving rise to diacylglycerol and an inositol phosphate glycan. In liver, muscle and fat cells, this hydrolysis reaction is acutely stimulated by insulin. The resulting inositol glycan modulates the activities of several insulin-sensitive enzymes involved in metabolic regulation, and mimics certain of the actions of insulin in intact cells. The activities of many, if not all of these enzymes are modulated by changes in the state of phosphorylation. Interestingly, the actions of this compound in fat and liver cells can be attenuated by okadaic acid, a protein serine phosphatase inhibitor. Preliminary structure/function studies on the inositol glycan have begun, using a combination of chemically synthesized compounds, as well as fragments obtained from the structurally defined glycosyl-PI anchor of the trypanosoma brucei Variant Surface Glycoprotein. Kinetic and mechanistic studies on the generation and action of the inositol glycan suggest a possible role as a second messenger for some of the acute actions of insulin. Elucidation of the functional role of glycosyl-PI in the generation of second messengers may provide further insights into the pleiotropic nature of the action of insulin and other hormones.

GPI-specific phospholipase D: a plasma enzyme that degrades the membrane anchor of cell surface proteins.

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Glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD) is a metal-ion dependent enzyme (molecular mass 100-110 kDa) found in mammalian plasma which is capable of cleaving the GPI anchor of several cell surface proteins. It has been proposed that the physiological role of this enzyme is to release GPI-anchored proteins from membranes thereby regulating their expression at the cell surface. Evaluation of this hypothesis requires detailed information on the identity of the physiologically relevant substrates, as well as the regulatory mechanisms and the cellular sites of synthesis and action of GPI-PLD. Recent work in this laboratory has been directed toward obtaining this information. Previous attempts to determine the tissue/cell localization of GPI-PLD by biochemical techniques have been compromised by the abundance of GPI-PLD in the plasma as well as the presence of competing enzyme activities. Monoclonal antibodies raised against GPI-PLD were therefore used to localize the enzyme in frozen sections of perfused rat tissue. Positive staining was obtained in stratified squamous epithelium in several locations (e.g. upper gastrointestinal tract, epidermis etc.) the cell bodies of motor neurons (spinal cord and brain) and sensory neurons of the dorsal root ganglion. A similar analysis of GPI-PLD distribution in embryonic rat tissues is currently being performed.

Potential mechanisms of GPI-PLD regulation are also under investigation. It was previously shown that GPI-PLD in plasma or serum is unable to release GPI-anchored proteins from cell membranes but the mechanism for this resistance is unknown. Studies with purified bovine serum GPI-PLD and reconstituted membranes suggest that protein factors in the serum or on the membranes are not responsible for the inability of GPI-PLD to act on membranes. The results predict the existence of some mechanism for activating the GPI-PLD and the potential role of proteolytic cleavage of the GPI-PLD polypeptide is currently being studied.

2nd SESSION

Insulin and IGF-1 induce the generation of inositolglycans in the extracellular medium

Studies on the mechanism of generation of inositolglycan mediators have suggested that insulin may physiologically induce the generation of these compounds in the extracellular medium. This hypothesis has been tested using anti-inositolgycan antibodies specifically developed with this purpose. These antibodies were raised against the soluble form of *Trypanosoma brucei* variant surface glycoprotein (VSG). *In vitro* tests demonstrate that the purified antibodies recognize specifically the oligosaccharide moiety of the glycosyl-phosphatidylinositol anchor of VSG. Biochemical tests demonstrate that these antibodies inhibit the *in vitro* functions of preparations of inositolglycan mediators of various degrees of purity. When used in intact cell assays, these antibodies were shown to block the activation of pyruvate dehydrogenase in BC_3H1 cells by insulin. Additional studies using swine ovary granulosa cells and human cytotrophoblasts have shown that the antibodies interfere with the effects of insulin and IGF-1 in the regulation of steroid hormone synthesis in intact cells. Both IGF-1 and insulin stimulate the rapid extracellular release of inositolglycan mediators from granulosa cells. These findings suggest a paracrine/autocrine mechanism of action of insulin and IGF-1 in which inositolglycan mediators play the role of autocrine factors. In agreement with this mechanism, the regulation of progesterone synthesis by insulin and IGF-1 was found to be strongly dependent on the cell density of the system. Quantitative analysis of the cell density dependence of the response suggests that the simplest model consistent with the data is a simple autocrine mechanism in which the soluble factor equilibrates with the extracellular medium. The implications of this mechanism are discussed.

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PHOSPHOLIPID METABOLISM AND PROTEIN KINASE C ACTIVATION IN INSULIN ACTION

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Insulin rapidly perturbs phospholipid metabolism in many target tissues, i.e., increases in: inositol-lipid synthesis; *de novo* phosphatidic acid (PA) synthesis; hydrolysis of phosphatidylinositol (PI)-glycan; and phosphatidylcholine (PC) hydrolysis. Insulin-induced hydrolysis of the PI-glycan and increases in *de novo* PA synthesis are inhibited by pertussis toxin, whereas PC hydrolysis is not. Increased *de novo* PA synthesis is due to an activation of glycerol-3-phosphate acyltransferase, by headgroup mediators (IPG) released from the PI-glycan. Thus, insulin activates two major phospholipid systems, viz., PI-glycan and PC hydrolysis, presumably via G-proteins coupling the insulin receptor to phospholipases. PI-glycan hydrolysis in turn activates *de novo* PA synthesis, which maintains PC levels. Both the *de novo* pathway and PC hydrolysis provide PA to synthesize PI, PIP, PIP_2 and the PI-glycan. These integrated pathways provide for rapid hydrolysis and re-synthesis of PC and the PI-glycan, and for generation of mediators, IPG and diacylglycerol (DAG). Increases in DAG derive from multiple sources during insulin action, i.e., PC hydrolysis, *de novo* PA synthesis and PI-glycan hydrolysis. DAG from each of these sourcesactivates protein kinase C (PKC), probably in different subcellular locations, involving specific PKC isoforms, and at different efficiencies. PKC activation is characterized by its translocation from the cytosol to DAG-enriched membranes. Using immunoblotting to detect changes in PKC content, or using Mono Q column chromatography to remove PKC modulators prior to enzyme assay, typical PKC translocation patterns are seen in rat adipocytes, rat soleus muscle, BC3H-1 myocytes, and H411E cells and rat gastrocnemius. Further evidence for PKC activation in intact tissues is suggested by the finding that insulin and PKC-activating phorbol esters provoke comparable phosphorylation of PKC substrates, e.g., 40, acidic 80 (MARCKS), 15 kDa and other proteins.

A potentially important role for DAG/PKC signalling in insulin-stimulated glucose transport is suggested by several lines of evidence. First, activation of PKC by various means uniformly provokes increases in glucose transport in many cell types. Second, all PKC inhibitors inhibit insulin-stimulated glucose transport. Third, prolonged treatment of rat adipocytes with phorbol ester, insulin and/or 20 mM glucose leads to PKC depletion, inhibition of insulin-stimulated glucose transport, but no compromise in earlier steps of insulin action. Contrasting findings have been noted in BC3H-1 myocytes, in which prolonged phorbol ester treatment results in inhibition of TPA, but not insulin, effects on glucose transport. In BC3H-1 myocytes, however, phorbol esters deplete PKC-α, but fail to diminish immunoreactive PKC-β, PKC-ε, PKC-ζ and PKC-dependent phosphorylation of non-histone substrates. Thus, considerable functional PKC is retained in these "downregulated" cells. In keeping with the latter, DAG- and phospholipase C-stimulated glucose transport remain intact in downregulated myocytes, and effects of insulin are sensitive to PKC inhibitors. In comparing the downregulation paradigm in rat adipocytes and BC3H-1 myocytes, it is intriguing that PKC-β is more effectively depleted by phorbol esters in adipocytes. Perhaps phorbol ester effects on glucose transport are primarily mediated by PKC-α, and insulin, primarily by PKC-β. Fourth, antisense-DNA targeted against mRNA's for PKC-β and PKC-α specifically downregulate these, but not other, PKC isoforms in rat adipocytes, and impair insulin and phorbol ester effects on glucose transport. Fifth, we have fully restored insulin effects on glucose transport in TPA-downregulated, PKC-depleted rat adipocytes by electroporation in the presence of pure PKC. The restoration of insulin effects by PKC replenishment indicates that PKC is required for, and may mediate, insulin effects on glucose transport.

Biological effects of glycosyl inositol-phosphate on adipocytes and thyrocytes : Role of pertussis toxin sensitive G proteins

by C. JACQUEMIN. *

We previously described the synthesis and release of glycosyl inositol-phosphate (InP-gly) from thyrotropin-(TSH-) stimulated polarized thyrocytes. This substance was characterized by its labelling with putative precursors and isolated according to its chromatographic properties.

We have studied the biological activity of InP-gly on fat-cells and compared its inhibitory character on cAMP accumulation with that of prostaglandin E2 and adenosine.

The IAP component of pertussis toxin blocked the action of the three negative agonists on cAMP accumulation promoted by isoproterenol, suggesting the participation of Gi protein in the transducing process.

We have also examined the autocrine activity of InP-gly on thyroid metabolism. The cAMP accumulation promoted by TSH or forskolin was modulated by InP-gly, stimulated by the lowest tested concentration (10^{-8}M) and progressively inhibited by higher concentrations. Iodide uptake and iodine organification were decreased in a concentration-dependent manner by InP-gly alone or in the presence of TSH.

In thyrocytes, IAP blocked the inhibitory action of InP-gly on cAMP accumulation increased by TSH. But the same treatment with IAP was without effect on iodine metabolism, suggesting that there is a second target for InP-gly, more distal than Gi protein, or coupled to another G protein which is insensitive to the toxin.

Tentative models will be presented.

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3rd SESSION

Structure and function of NGF receptors. Moses V. Chao, Department of Cell Biology & Anatomy, Cornell University Medical College, 1300 York Avenue, New York, New York USA 10021

The many differentiative and survival effects of nerve growth factor (NGF) on responsive cells are directly mediated by binding to high affinity cell surface receptors. NGF interacts with two different receptor proteins that can be distinguished by affinity crosslinking with ^{125}I-NGF. Reconstitution experiments by membrane fusion and transfection into heterologous cells indicate that high affinity NGF binding requires co-expression and binding to both the low affinity NGF receptor ($p75^{NGFR}$) and the product of the proto-oncogene *trk* ($p140^{prototrk}$), a receptor tyrosine kinase. NGF is capable of binding with low affinity to $p140^{prototrk}$ alone, and can trigger autophosphorylation activity. Mutations made in the highly conserved cytoplasmic domain of the p75 NGF receptor abolished high affinity site formation, and also eliminated the ability of NGF to elicit tyrosine phosphorylation. These studies reveal a novel growth factor receptor-mediated mechanism of cellular *differentiation*, involving the tyrosine kinase *trk* and the low affinity NGF receptor. A direct prediction from the two NGF receptors is that specificity of neurotrophic action must be directly mediated by the expression of an appropriate *trk* tyrosine kinase in concert with $p75^{NGFR}$. Additionally, the identification of the proto-oncogene *trk* as an NGF receptor provides evidence for the primary signaling mechanism of the neurotrophic family of growth factors.

ROLE OF THE GLYCOSYL-PHOSPHATIDYLINOSITOL/ INOSITOL PHOSPHOGLYCAN SIGNALLING SYSTEM IN THE REGULATION OF GENE EXPRESSION AND CELL PROLIFERATION

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The activation of insulin-, NGF-, EGF-, TSH- and IL-2 receptors leads to the hydrolysis of a membrane glycosyl-PtdIns. The products of this reaction are inositol phosphoglycan (IPG) and diacylglycerol. IPG has been shown to mimic a variety of the short-term effects of insulin. We have studied the possible effect of the glycosyl-PtdIns/ IPG system on cell proliferation in insulin-dependent and independent models and the role of IPG in the insulin-like regulation of the expression of specific mRNAs. The biological significance of IPG on cell proliferation was investigated by studying its effects in the early developing inner ear. IPG has insulin mimetic effects on the growth and morphogenesis of the otic vesicle potentiating the mitogenic effect of bombesin. In the cochleovestibular ganglion the role of the glycosyl-PtdIns/ IPG pathway in transducing the mitogenic effects of NGF has been investigated by showing: i) The presence of endogenous glycosyl-PtdIns and IPG, the later with strong mitogenic activity, ii) The ability of NGF to stimulate glycosyl-PtdIns hydrolysis in parallel with its biological activity, and iii) The ability of anti-IPG antibodies to block the biological effects of NGF. The ability of IPG to mimic insulin effects on the regulation of the expression of specific mRNAS was studied in isolated hepatocytes from normal and diabetic rats. Incubation of normal liver cells with IPG produced a decrease in phosphoenolpyruvate carboxykinase (PEPCK) mRNA levels, which had been previously increased by incubation with 8-Br-cAMP. IPG reduction of PEPCK mRNA is primarily due to a decrease in the rate of transcription of the gene as judged by nuclear run-on transcription experiments performed in rat hepatoma H4IIE cells. In hepatocytes isolated from diabetic rats, treatment with IPG caused an induction in the expression of α2-microglobulin mRNA, concomitantly with a decrease in the level of PEPCK mRNA. These data indicate that both, positive and negative, effects of insulin on the regulation of gene expression are mimicked by IPG.

REGULATION OF T LYMPHOCYTE GROWTH AND FUNCTION BY GPI

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Glycosylated-phosphatidylinositol (GPI) molecules have been implicated as precursors for both hormone-sensitive second messengers and lipid anchored membrane proteins. The relationship between the diverse functions of these lipids and predicted structural heterogeneity within GPI subtypes was examined in human and murine T lymphocytes. Eight subtypes of GPI molecules were identified in T cells following separation over 2-dimensional high performance thin layer chromatography (HPTLC), by sensitivity to phosphatidylinositol-specific phospholipase C (PI-PLC) and nitrous acid. Limited structural analysis of mixed GPI populations indicated that this heterogeneity is associated with variation in glycan size and content, and fatty acid composition and linkage. Hydrolysis of lymphocyte GPI was observed in response to both insulin and interleukin-2 binding. Antibody probes of the glycan domains of GPI were developed and used to assess the partial sensitivity of GPI to hormone action. This analysis showed that the effects of insulin and interleukin-2 are linked to differential utilization of a subset of GPI molecules. The polar fragments of this reaction were identified in extracellular supernatants from hormone treated cells. The biological significance of insulin and interleukin-2 dependent GPI hydrolysis was demonstrated by insulin, interleukin-2 and IPG regulation of glucose metabolism in intact lymphocytes. These results support the hypothesis that the multifunctional roles of GPI are served by discrete GPI populations and that the metabolites of GPI subsets participate as signaling elements in hormone action and the regulation of T cell growth.

4th SESSION

Regulation of glucose transport in fat cells: possible role of inositolphosphooligosaccharides.

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Insulin activates the glucose uptake of isolated fat cells through translocation of the glucose transporter isoforms GLUT4 and GLUT1 from intracellular membranes to the plasma membrane. The effect of insulin on GLUT4, but not GLUT1 can be mimicked by phorbolesters and other pharmacological tools. While phorbolesters are able to mimic the full insulin effect on GLUT4 translocation only a partial activation of the glucose transport system is observed suggesting that insulin acts through a second mechanism activating glucose carriers in the plasma membrane. This activating effect of insulin, independent of carrier translocation can be mimicked by exogenously added phospholipase C, aluminiumchloride and insulin-like acting compounds isolated from haemodialysate of calf blood (Actovegin). There is some evidence that the active compounds of these fractions are inositolphosphooligosaccharides. These putative IPO-fractions exert in isolated fat cells the following effects: activation of 3-O-methylglucose uptake, decrease of catecholamine stimulated cyclic AMP-levels and activation of lipogenesis. The effect of these IPOs on lipogenesis in fat cells is blocked by the addition of mannose and glucosamine suggesting a mannose- and glucosamine-specific uptake mechanism. To test whether analogous compounds are released by fat cells after insulin stimulation we applied the same purification procedure which was developed for calf blood haemodialysate to isolate insulin-like acting compounds from the medium of insulin stimulated fat cells. By this approach we found indeed insulin-like activity in the supernatant of insulin stimulated fat cells. Subsequently the same technique was applied to test whether rat-1 fibroblasts overexpressing either the insulin receptor isoform A (HIR-A) or the receptor isoform B (HIR-B) release IPOs as well. In analogy to the fat cell experiments insulin stimulated release of putative IPO fractions was observed after stimulation of both cell types. This putative IPOs released by rat-1 fibroblasts were active in the bioassay on 3-O-methylglucose uptake and lipogenesis of isolated fat cells. The data suggest that fibroblasts are able to release IPOs which play a role in a cell/cell signalling system. This effect can be triggered by both insulin receptor isoforms.

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Inositol-glycan and Diacylglycerol: Dual Second Messengers of Insulin?

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The possibility that the short-term actions of insulin are mediated by the dual second messengers inositol-glycan (IPG or POS for phosphooligosaccharide) and diacylglycerol (DAG) is considered. It has been shown that, upon insulin stimulation of cells, soluble IPG is generated in concert with long-chain diacylglycerol.

I will demonstrate that in intact adipocytes IPG elicits a phosphorylation response pattern that is indistinguishable from that of insulin: insulin effects by itself or the ability of insulin to counteract effects of cAMP-elevation (*Nature* 330, 77-79). It seems that IPG can mediate the metabolic control of insulin through control of the state of phosphorylation of target proteins. The highly polar IPG molecule will have to reach the interior of the adipocytes through some kind of transport over the plasma membrane. I will demonstrate that compounds structurally related to IPG from the out-side block the insulin-like effects of IPG in adipocytes, and, most importantly, the same compounds also block the effects of insulin itself on protein phosphorylation (*FEBS Lett.* 268, 169-172). These findings are taken to indicate that IPG is generated on the cell surface and transported over the plasma membrane as part of the hormone's physiological signal transducing pathway.

I will present evidence suggesting that the function of insulin to promote glucose transport in eg adipose tissue can, at least partly, be provided for by DAG and apparently so without involving protein kinase C. In isolated adipocytes certain exogenously added long-chain DAG, but nor short-chain DAG, enhance glucose transport (*Nature* 335, 554-556). Dimyristoylglycerol was found to have an optimum chain length and saturation for stimulation of glucose uptake. This diacylglycerol does, however, not affect protein kinase C activity in the cells. Dioctanoylglycerol on the other hand potently activates protein kinase C, but is unable to stimulate glucose transport. The ability of DAG to stimulate glucose transport in different cells correlates with the expression of the insulin-sensitive glucose transporter GLUT-4. In hepatoma cells (H4), that are responsive to insulin, but does not express GLUT-4, glucose transport is neither affected by insulin nor by dimyristoylglycerol. The DAG seems to affect the activity of the glucose transporter rather than its translocation to the plasma membrane. The translocation process are affected by agents known to control protein phosphorylation (eg: phorbol esters, okadaic acid) which suggests that insulin may control this process through the IPG.

INFLUENCE OF STREPTOZOTOCIN-DIABETES AND DEXAMETHASONE-INDUCED INSULIN RESISTANCE ON GLYCOSYL-PHOSPHATIDYLINOSITOL LEVELS AND INOSITOL-PHOSPHOGLYCAN UPTAKE IN ISOLATED RAT HEPATOCYTES.

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It is now generally accepted that after the interaction with its receptor, insulin promotes the hydrolytic cleavage of a glycosyl-phosphatidylinositol (GPI) by a specific phospholipase C, generating an inositol-phosphoglycan (IPG). This compound, of partially known chemical structure, has been demonstrated to modulate the activity of certain insulin-sensitive enzymes.

In agreement with several reports which suggest a cellular uptake of IPG, we have recently reported the existence of an IPG transport system in isolated rat hepatocytes which is specific, and energy-, time- and dose-dependent (1).

Nevertheless, little is known about the modulation of the cellular levels of glycosyl-PI and the rate of hepatocyte IPG uptake in metabolic states associated with insulin resistance. In recent studies (1,2), we have demonstrated that the administration to rats of dexamethasone, which causes insulin resistance, provokes a marked reduction in hepatocyte glycosyl-PI levels, a suppression of the hydrolytic cleavage of this molecule in response to insulin, a decrease in the rate of IPG uptake and a blockade of the stimulatory effect of this compound on glycogen synthesis. Similar changes were also demonstrated in hepatocytes isolated from either streptozotocin-diabetic rats or 24-mo-old rats (3).

The fact that different states of insulin resistance cause a comparable impairment of the glycosyl-PI-dependent signalling system suggests that this could be the common consequence of an altered insulin action at the cellular level and possibly not the primary defect.

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5th SESSION

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GPI acts as an apical targeting signal in polarized epithelial cells, Enrique Rodriguez-Boulan, Department of Cell Biology, Cornell University Medical College, New York

We have recently proposed a role for the glycosyl phosphatidylinositol (GPI) anchor in protein trafficking (5,14). Specifically, we have presented evidence indicating that GPI is responsible for the apical localization of proteins anchored via this mechanism in epithelial cells (4). The evidence is the following: (1) Using a biotin polarity assay, all GPI-anchored proteins detected are apical in epithelial cell lines of kidney (MDCK, LLCPK) and intestinal (Caco-2, SK-CO15) origin (6,10); (2) Transfection of GPI-proteins normally not found in epithelial cells rsults in apical localization (13); (3) Transfer of GPI by recombinant DNA procedures results in apical localization of unsorted secreted or basolateral membrane proteins (4), the apical targeting role of GPI does not depend on remaining aminoacids from the GPI-attachment signal (8); (4) Transfection into kidney MDCK cells of three isoforms of the neural adhesion molecule N-CAM, one GPI-anchored and two anchored by conventional hydrophobic transmembrane segments results in the apical localization of GPI-N-CAM and in the basolateral localization of the transmembrane forms (12); (5) treatment of MDCK cells expressing a transfected apical GPI-anchored protein, gD1-DAF, with a novel inhibitor of GPI-anchoring, mannosamine, results in the non polarized secretion of gD1-DAF from both epithelial surfaces (9). We have also shown that the delivery of GPI anchored proteins to the apical surface occurs directly from the Golgi complex by a vectorial process (3,7) and is inhibited by agents that disrupt the microtubules (1). Very recently we have observed that a concanavalin A resistant ($ConA^r$) mutant of MDCK cells (11) fails to sort properly gD1-DAF, which appears unpolarized on both apical and basal surfaces (Lisanti and Rodriguez-Boulan, unpublished observations). We hypothesized that MDCK-$ConA^r$ express a mutant phenotype for apical targeting of GPI-proteins. We tested the hypothesis that GPI patching may be involved in apical targeting (6) using fluorescence recovery after photobleaching (FRAP) and fluorescence energy transfer. GD1-DAF recently arrived to the cell surface from the Golgi apparatus was found to be aggregated in wild type cells but fully diffusible in MDCK-$ConA^r$, suggesting that aggregation in the Golgi apparatus may be responsible for preferential delivery to the apical surface (2). We are currently searching for Golgi "sorting receptors" that may be involved in this process. Supported by grants from NIH

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Expression and release of glycosyl-phosphatidylinositol anchored molecules during neuronal differentiation.

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The study of the molecular bases of the cell interaction mechanisms has recently led to the identification of a number of cell surface glycoproteins mediating recognition events in the nervous tissue. A shared feature of several of these molecules is their anchorage to plasma membrane by a glycosyl-phosphatidylinositol (GPI) group.

The first part of my talk will deal with the cloning, biochimical characterization, cell and tissue expression of two such molecules we recently identified and termed HSA/P31 and F3 respectively.

HSA/P31 is a cell surface glycoconjugate which is transiently expressed on neurons during development and on subsets of cells of the hematolymphoid lineage (1). The differences of molecular weights of the antigens expressed by these two lineages reflect a specialization for each cell-type resulting from differential glycosylation of a common protein backbone (2). The protein core has been cloned (3) and the cDNA encodes a surprisingly small peptide predicted to contain only 30 amino-acid residues for the mature protein. In developing mouse brain, this glycoconjugate is expressed by post-mitotic neurons during a period that corresponds to cell migration and formation of neuronal networks (4).

F3 is a new member of the immunoglobulin super-familly of 135 kD molecular weight (5). F3 antigen expression undergoes developmental regulation since the concentration in brain extracts is maximun during the two first postnatal weeks (6). In cell culture as well as on brain tissue sections F3 was found to be confined to subsets of neurons and appeared to be, on some of them, polarized to the axons (7). In vitro assays using F3 transfected CHO cells indicated that F3 promotes neurite outgrowth and could be involved in heterophilic adhesion mechanisms (8).

In the second part of my talk I will describe studies on expression and release of GPI-anchored molecules by a cell line derived from sensory neurons whose differentiation state could be controlled by manipulating the composition of the culture medium (9). This cell line expresses NCAM120 isoform, F3, Thy1, F3, cellular isoform of scrapie prion and P31, all GPI-anchored molecules, whose expression is developmentally regulated. We found that NCAM120 and expression decreased upon differentiation whereas the level of expression of the other GPI-anchored molecules increased suggesting they play a role in neurite outgrowth processes.

The measurement of the quantities of GPI-molecules spontaneous released in the culture medium indicated that they reflect the overall level of expression of these molecules by the cells.

Finally we identified two GPI-phospholipase activities associated with particulate membrane fractions and differing by their pH activity maxima and sensitivity to activators and ion chelators (10). Such enzymes are likely to be involved in the release of GPI-anchored molecules from the plasma membrane.

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INOSITOL GLYCANS AS INSULIN MIMETICS: STRUCTURAL STUDIES AND NEW SYNTHETIC APPROACHES

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The structures of the low molecular weight mediators which seem to act as intermediates in the intracellular insulin signaling process have not yet been completely elucidated. Mato et al[1] and Larner et al[2] have reported similar but different chemical compositions of species of partially purified inositol glycans from rat liver which mimick insulin action. A type of inositol glycan seems to contain *chiro* and *myo*-inositol, non N-acetylated glucosamine, galactose and phosphate whereas a second class seems to be composed of D-*chiro* inositol, non N-acetylated galactosamine, mannose and phosphate. The determination of the complete structure of these mediators has been hampered by the minute amounts which can be obtained from biological material which prevents the application of conventional chemical and spectroscopic methods with an aceptable degree of reliability.

The important amount of relevant literature on the glycosyl phosphatidyl inositols[3] which have been proposed to anchor protein, polysaccharide, or small oligosaccharides to cellular membranes, and the elucidation of the complete structure of two of these anchors[4,5] have provided a body of useful information which may be applicable to the structural determination of these putative insulin mediators.

Starting from bovine liver a workable amount of active material has been obtained and investigations on the structure(s) of the active product(s) are in progress. On the other hand, two different synthetic approaches to *myo*- and D-*chiro* inositol-containing glycans have been developed which may lead to taylor-made glycosylphosphatidyl inositols in reasonable yields and in sufficient amount as to permit a series of biological assays.

The structural and synthetic work will be presented and a survey of the results reported by other groups working in the same area will also be given.

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POSTER SESSIONS

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p97 (MELANOTRANSFERRIN) IS GPI-ANCHORED AND APICALLY LOCALIZED IN INTESTINAL EPITHELIAL CELLS.

Melanotransferrin is a monomeric membrane bound glycoprotein with aminoacid sequence homology to human transferrin and lactoferrin which also has the capacity to bind iron. Although it was first identified as a cell surface 97 KD glycoprotein in cultured human melanoma cells, it has been later found on a wide range of cultured cell types including kidney and intestinal epithelial cell lines. In normal tissues, melanotransferrin (p97) on normal tissues can be detected in fetal intestine, umbilical cord, sweat gland ducts and in sinusoidal lining cells of the liver. Studies on iron uptake in melanoma cells suggest that p97 plays a role in iron metabolism. To further clarify its function, we have examined the distribution of p97 on intestinal epithelial cell lines Caco 2 (enterocytic), SK-Co 15 (undifferentiated but polarized) and HT29 MTX (mucus-secreting by growth adaptation to methotrexate). It has been detected in all of them by metabolic labelling and immunoprecipitation. Furthermore, when its subcellular distribution was examined by immunocytochemistry and selective biotinylation, it was found to be apically polarized. As GPI-anchor has been described for targetting proteins to the apical cell surface and no clear cytoplasmatic tail has been identified in p97, cells were treated with PI-PLC and p97 was found exclusively in the supernatant, indicating that p97 is GPI-linked.

The polarized distribution of p97 in intestinal cells and its GPI-anchor suggest that melanotransferrin is involved in iron transport in this tissue. Functional studies are currently underway to answer this question.

INSULIN-LIKE EFFECTS OF INOSITOL PHOSPHATE-GLYCAN ON mRNA EXPRESSION IN RAT HEPATOCYTES

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The ability of an inositol phosphate-glycan (IPG) to mimic insulin effects on the regulation of the expression of specific mRNAs was studied in isolated hepatocytes from normal and diabetic rats. Incubation of normal liver cells with IPG (10 µM) during 90 min produced a 5-fold decrease in phosphoenolpyruvate carboxykinase (PEPCK) mRNA levels, which had been previously increased about 10-fold by incubation with 8-Br-cAMP (0.1 mM). IPG effect was dose dependent, and could not be reproduced by galactose, glucosamine or *myo*-inositol. IPG reduction of PEPCK mRNA is primarily due to a decrease in the rate of transcription of the gene as judged by nuclear run-on transcription experiments performed in rat hepatoma H4IIE cells. In hepatocytes isolated from diabetic rats, treatment with 5 µM IPG for 15 min caused a 4-fold induction in the expression of α2-microglobulin mRNA, concomitantly with a 2.5-fold decrease in the level of PEPCK mRNA. Cleavage of IPG with nitrous acid abolished both the increase and decrease in specific mRNAs levels. Glycosyl-phosphatidylinositol, the lipid precursor of IPG, did not modify either PEPCK or α2-microglobulin mRNA levels. These data indicate that both, positive and negative, effects of insulin on the regulation of gene expression are mimicked by IPG.

ENZYMATIC PROPERTIES OF PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C FROM *BACILLUS CEREUS* AND MAMMALIAN PHOSPHATIDYLINOSITOL-GLYCAN-SPECIFIC PHOSPHOLIPASE D

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In the last few years, a large number of phosphatidylinositol-glycan (PtdIns-glycan)-anchored proteins and glycolipids have been identified. The bacterial PtdIns-specific phospholipase C (PI-PLC) cleaves phosphatidylinositol (PI) as well as PtdIns-glycan-anchored proteins while in eucaryotic cells, two distinct enzymes which specifically convert the membrane forms of PtdIns-glycan-anchored molecules to the soluble form, have been described: PtdIns-glycan-specific phospholipase C (PIG-PLC) which occurs in *Trypanosoma brucei* and PtdIns-glycan-specific phospholipase D (PIG-PLD) which is found in relatively high amounts in mammalian sera (bovine >> human ≥ swine). Although the enzyme appears to be soluble in serum, it displays amphipathic properties as revealed by sucrose densitiy gradient centrifugation in absence and presence of detergent. In order to compare the bacterial PI-PLC to mammalian PIG-PLD in identical assay conditions, we investigated the enzymatic properties of PI-PLC from *Bacillus cereus* and PIG-PLD from mammalian sera towards pure PtdIns-glycan-anchored acetylcholinesterase (AChE) from bovine erythrocytes as substrate. The kinetic parameters showed marked differences between the bacterial and the mammalian enzyme. PI-PLC converted AChE with a K_m of 17 µM while the K_m for PI was determined at 2 mM. PIG-PLD from mammalian sera had a K_m around 0.1 µM for AChE and did not recognize PI as substrate. The differences in substrate specificity and in K_m-values indicated that the glycan moiety of AChE is an essential part for substrate binding. PI-PLC was not Ca^{2+}-dependent and not inhibited by chelating agents while PIG-PLD gave a large increase in activity between 0.1 µM and 10 µM Ca^{2+} indicating that PIG-PLD is only marginally active at physiological intracellular Ca^{2+}-concentrations. PIG-PLD was inhibited by heavy metal chelating agents such as 1,10-phenantroline and 2,2'-bipyridyl but not by the corresponding Fe^{2+}-complexes or non-chelating analogues indicating that it requires a heavy metal ion for the expression of catalytic activity in addition to Ca^{2+}. Another interesting property of PIG-PLD is its inactivation by bicarbonate and cyanate. The inactivation was time and pH-dependent and could be reversed by dialysis. These observations are in agreement with a covalent modification of the enzymes by carbamoylation.

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GLYCOSYLPHOSPHATYDYLINOSITOL/INOSITOL PHOSPHOGLYCAN: A TRANSDUCING SYSTEM IN HUMAN FIBROBLAST PROLIFERATION.

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Several hormones and growth factors induce hydrolysis of a glycosylphosphatidylinositol (GPI) and subsequent release of its polar headgroup, an inositolphosphoglycan (IPG), in a large variety of cells (1). IPG, has been shown to reproduce in hepatocytes and adipocytes, some of the early biological effects of insulin, such as inhibition of lipolysis and gluconeogenesis and stimulation of lipogenesis (2,3). We have recently shown that IPG, similarly to insulin, is able to stimulate glycolysis in human fibroblasts (4).

In the present study human fibroblasts were investigated for the presence of an insulin sensitive GPI. A glycolipid fraction which could be labelled with [^{3}H]glucosamine was significantly reduced upon insulin stimulation of the cells indicating the hormonal sensitivity of the compound. The partial composition of the lipid was also examined by labelling cells with different precursors. The ability of the polar head group of the GPI to carry on fibroblast proliferation was also investigated and the effects of IPG were compared to those induced by insulin. The incubation of fibroblasts in the presence of insulin or IPG resulted in a significant stimulation of [^{3}H] leucine incorporation into cellular protein. The stimulatory effect of IPG on protein synthesis was similar to that exerted by the hormone also comparing the electrophoretic pattern of proteins from fibroblasts challenged with either IPG or insulin in the presence of [^{35}S] methionine. The compound was also able to increase [^{3}H] uridine and [^{3}H] thymidine incorporation into RNA and DNA. IPG increased these parameters in a dose dependent manner in the range 0.03-10 uM for both leucine and uridine incorporation, reaching a stimulation as high as 100% above controls, and in the range 0.1-10 μM for thymidine incorporation, that was

stimulated up to 10-fold over the controls. The specificity of these effects was controlled evaluating the action of different sugars and of IPG deaminated with nitrous acid. The effects of IPG on macromolecular syntheses are not mimicked or reduced by the single sugars and the structural integrity of IPG is required, since deaminated IPG is inactive.

The present results indicate that the hydrolysis of GPI could represent a major signalling pathway for the proliferative processes in human fibroblasts since the IPG molecule can stimulate significantly macromolecular syntheses in these cells.

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SYNTHESES AND INSULIN-LIKE ACTIVITY OF PHOSPHORYLATED GALACTOSE DERIVATIVES

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It has been reported that insulin stimulates the generation of at least two structurally different types of inositol-glycans with insulin-like activity[1, 2]. One of these inositol glycans seems to be composed of *myo* and/or *chiro*-inositol, glucosamine, galactose and phosphate[1]. As a part of a project on the synthesis of inositol-glycans with insulin-like activity, we have synthesized several phosphorylated galactose-containing mono, di- and tri-saccharide derivatives.

The syntheses of these compounds will be presented and their biological activity as insulin-mimetics will be reported.

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SIGNALLING AT THE EPIDERMAL GROWTH FACTOR RECEPTOR: ROLE OF GLYCOSYL-PHOSPHATIDYLINOSITOL

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Activation of epidermal growth factor (EGF) receptors induce a number of early intracellular events. This include activation of the intrinsic tyrosine kinase activity with phosphorylation of the receptor itself. It has been shown that this activity is an indispensable element in the chain of intracellular events which will lead to the stimulation of cell proliferation. However, it is incompletely understood how the mitogenic signals are transmitted from the receptor tyrosine kinase. The glycosyl-phosphatidylinositol/ inositol phosphoglycan (glycosyl-PtdIns/IPG) system has been proposed as a candidate for signal transducer of a variety of factors with either intrinsic (insulin & IGF-I) or associated (NGF & IL-2) tyrosine kinase activity.

NIH 3T3 cells which express a small number of EGF receptors, are poorly responsive to EGF. However, when the same cells overexpress the cloned human EGF receptor (EGFR T17 cells), they display EGF-dependent proliferation. We have characterised the endogenous glycosyl-PtdIns in both cell lines. In EGF T17 cells (but not in the parental NIH 3T3 cells), we have found that EGF stimulates glycosyl-PtdIns turnover. Hydrolysis was maximal when [^{3}H]glucosamine-labelled cells were treated with 50 nM EGF for 1 min. Inhibitors of the receptor tyrosine kinase activity (tyrphostins and genistein) affect the EGF-dependent hydrolysis of glycosyl-PtdIns. The possible role of IPG as signal transducer for EGF on EGFR T17 cells has been evaluated by showing the following results: i) the ability of IPG to mimic the proliferative effect of EGF; and ii) the ability of anti-IPG antibodies to partially block (60%) the biological effects of EGF.

FUNCTIONAL STUDIES AND LOCALIZATION OF F3 NEURONAL CELL SURFACE PROTEIN

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We previously identified, cloned and sequenced a GPI-anchored molecule F3 belonging to a subclass of proteins of the immunoglobulin superfamily with preferential localization on neurites in neuronal cell cultures (Gennarini et al. 1989 a, b).

We investigated the distribution of F3 in the adult and developing cerebellum by immunocytochemistry at the light and electron microscopic levels. F3 is confined to subsets of neuronal types. In developing cerebellum, the granular cell axons strongly express F3 as soon as they begin to grow, consistent with a functional role in promoting directional outgrowth of neuronal processes. In adult cerebellum, F3 expression is polarized on granule cells axons. F3 is found at only one side of the synaptic sites, as an indication that, if F3 were to be an adhesion molecule in vivo, it would mediate heterophilic interactions.

In vitro studies using F3-transfected cells as a culture substrate for sensory neurons confirmed the F3 ability to promote neurite outgrowth (Gennarini et al. 1991). Further investigations demonstrated that the soluble form of the molecule released from the membranes after PI-PLC cleavage, is responsible for the promoting effect. Moreover, in this model system, F3 acts via an heterophilic mechanism.

Our observations favored the hypothesis that GPI-anchored molecules may mediate activation signals by interacting with other signal transducing molecules.

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GLYCOSYLPHOSPHATIDYLINOSITOL: A CANDIDATE SYSTEM FOR PROLACTIN SIGNAL TRANSDUCTION IN OVARIAN GRANULOSA CELLS.

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Ovarian granulosa cells (GC) were cultured under serum free conditions and labeled with ^{3}H-glucosamine (Gln), ^{3}H-galactose (Gal), ^{3}H-myoinositol (Ins), ^{3}H-palmitate (Pal) or ^{3}H-myristate (Myr) for different time periods (6-72 hours). The lipids were extracted and a glycosylphosphatidylinositol (GPI) was separated by TLC from other phospholipids and characterized by: i)nitrous acid deamination ii)acid hydrolysis iii)PLA_2 (from bee venom) and iv)PLC (from S. aureus) treatment. These experiments revealed that the GPI in granulosa cells is not substantially different from those reported in the literature (1,2), and specifically incorporates labeled Gln, Gal and Ins into the polar head phosphooligosacharide (POS) and Pal and Myr in the positions 1 and 2 of the diacylglycerol (DAG) backbone respectively. Treatment of the cells with 30 ng/ml FSH (NIADDK-oFSH-S16), $(but)_2$cAMP (0.5 mg/ml) or CTX (0.5 μg/ml), induced GC differentiation, the development of functional receptors to PRL, and increased (6-10 fold) the GPI labeling with both ^{3}H-Gal and ^{3}H-Gln.

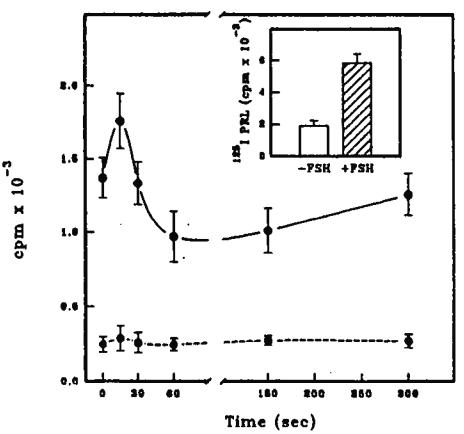

Fig. 1: Effects of PRL (1 μg/ml) on GPI turnover in (---) undifferentiated and (—) differentiated (pretreated with 30 ng/ml FSH) ovarian granulosa cells.

Addition of 1 μg/ml PRL (NIADDK-rPRL-B-5) to the cultures (Fig. 1), resulted in rapid (30 sec) and transient (5 min) changes in GPI turnover in differentiated granulosa cells. There was not effect on the GPI levels in undifferentiated cells, that do not express PRL receptors (Fig. 1 inset). Parallel experiments performed with ^{3}H-Ins labeled cells showed no effect of PRL on the turnover of phosphoinositides.

Differentiated cells labeled with ^{3}H-Myr accumulated ^{3}H-Myr DAG whitin 60 seconds in response to PRL. In another series of experiments (Fig. 2), GC were cultured in the presence or absence of FSH, $(but)_2$cAMP or CTX with and without PRL or POS, the latter obtained by treatment of the GPI extracted from rat liver with phosphatidylinositol specific PLC.

The GC steroidogenic activity, as assesed by the induction of 3ß hydroxysteroid dehydrogenase, and progesterone production, was inhibited by both PRL and POS in differentiated cells. The absence of PRL receptors in undifferentiated cells, prevents its biological effects, but POS was equally efective in these cells than in those previously exposed to FSH to obtain differentiation.

These results suggest that after binding to its receptor in GC membrane, PRL activates a GPI specific PLC, and that the subsequent release of the POS moiety mimics at least one of the PRL effects on steroidogenesis, this being the first report of an intracellular messenger for PRL in GC.

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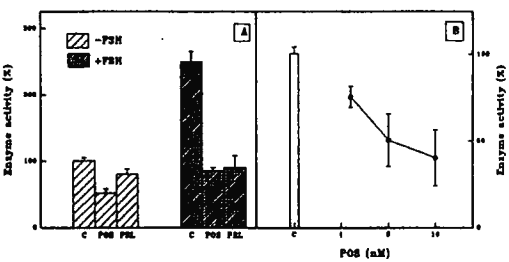

Fig. 2: Comparative effects of PRL (1 μg/ml) and POS (5 nM) on 3ß hydroxysteroid dehydrogenase activity (A), and dose response of POS (B).

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MECHANISM OF SIGNAL TRANSDUCTION ELICITED BY INTERLEUKIN 2 .

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Interleukin 2 (IL-2) plays a central role in the immune system regulating the proliferation and differentiation of T lymphocytes. However, the molecular mechanisms of the signal transduction through the IL-2 receptor are poorly understood. Recently it has been shown that IL-2 induces the release of inositolphosphoglycans (IPG) from a pool of glycophosphatidylinositol (GPI) lipids. The activation of a PI-specific phospholipase C (PLC) or D (PLC) could be the cause of this release. We have studied the role of phospholipid metabolism on IL-2 signal transduction using cloned T lymphocytes. The addition of exogenous PLD but not PLC mimicked IL-2 activity. Thus, PLD was able to induce DNA synthesis in cloned T cells, although this effect was not as potent as the one observed with IL-2. In addition, PLD was able to induce c-myc RNA synthesis in T cells as well as IL-2R receptor (CD25) expression on T cell membrane with equal potency as saturating doses of IL-2. Furthermore, IL-2 stimulated a transient increase in phosphatidic acid (PA) content in resting CTLL-2 cells prelabelled with (3H) palmitic acid. This effect was detected as early as 1 minute after IL-2 addition and peaked at 5 minutes. Furthermore, exogenous addition of several natural or synthetic PAs mimicked IL-2 activity to the same extent as PLD. Thus, PAs were able to induce c-myc RNA synthesis as well as IL-2R receptor (CD25) expression and partially restore T cell growth. Similar results on IL-2R expression were obtained with purified IPG obtained from PLD hydrolysis of GPI-lipids. However, IPG was unable to induce CTLL2 proliferation by itself unlike PA although it was synergistic in T lymphocyte proliferation with IL-2 as PA. In summary, our results suggest a role of PLD activation and hydrolysis of GPI-molecules rendering IPG and PA as second messengers of IL-2 activity.

LOSS OF EXTRACTIBILITY OF GPI-ANCHORED PROTEINS DURING THEIR TRANSPORT TO THE PLASMA MEMBRANE IN CACO$_2$ CELLS.

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We have shown that in intestinal epithelial cells in vitro proteins expressed on the apical membrane are sorted either at the Golgi or at the basolateral membrane level. Among these proteins GPI-anchored proteins (CEA, Alkaline phosphatase PLAP) are directly transported from the Golgi to the apical membrane and are transcytosed poorly [Le Bivic et al. 1989,1990]. Taken together with the fact that GPI-anchored proteins are preferentially localized on the apical membrane in intestinal cells [Lisanti et al. 1990] there might be a role for the GPI anchor in the specific sorting and transport of these proteins. We have investigated the possibility of interaction of GPI-anchored proteins with lipid microdomains in the Golgi apparatus. After a short metabolic pulse Caco-2 cells grown on filters were extracted under different conditions and with different detergents. We found that CEA and PLAP become resistant to Triton X 100 extraction when they are processed by the Golgi while a transmembrane apical protein such as sucrase-isomaltase (SI) or a basolateral protein (Ag525) remain extractable in the same conditions. The Triton X 100 insoluble pool of CEA and PLAP can be solubilized by octylglucoside suggesting that this pool may be formed by GPI-anchored protein enriched domains of specific lipid composition. We are currently purifiyng these membrane domains to study their composition and their role in the sorting of GPI-anchored proteins in epithelial cells.

SYNTHESIS OF CRUCIAL OPTICALLY PURE INTERMEDIATES FOR THE SYNTHESIS OF CONGENERS OF THE NATURALLY OCCURRING POLY-PHOSPHOINOSITIDES

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In animal cells there are several myoinositol containing phospholipids: phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP_2) etc... They are very active metabolically. They usually account for less than 10% of the total phospholipids in mammalian cell membranes.

"AGONIST STIMULATED PHOSPHOLIPASE C CLEAVES PIP_2 INTO TWO SECOND MESSENGER PRODUCTS; IP_3 AND DIACYLGLYCEROL (DG)".

The former mobilizes calcium from intracellular and extracellular pools, whereas DG activates protein kinase C. Further hydrolysis of DG generates arachidonic acid (AA), which is also an intracellular messenger. These messenger systems control a vast array of physiological processes.

In any event, it is clear that the PI cascade is the main source (obviously not exclusively) of the **"INTRACELLULAR AND EXTRACELLULAR CALCIUM, DIACYLGLYCEROL AND ARACHIDONIC ACID."**

THEREFORE IT IS CONCEIVABLE THAT CELLULAR EVENTS, WHICH ARE INTIMATELY ASSOCIATED WITH PHOSPHOINOSITIDE, PROSTAGLANDIN and LEUKOTRIENE CASCADES AND ALSO WITH PAF INDUCED SIGNAL TRANSDUCTION, *CAN BE ALTERED BY AGONISTS AND ANTAGONISTS AT BOTH BIFURCATING SIGNAL PATHWAYS OF THE PHOSPHOINOSITIDE CYCLE.*

A major challenge is now the elucidation of molecular aspects of the interaction of $Ins(1,4,5)P_3$ with its receptor and with the enzymes involved in its metabolism; i.e. $Ins(1,4,5)P_3$-kinase and 5-phosphatase, and the chemical design of novel $Ins(1,4,5)P_3$ agonists, antagonists and enzyme inhibitors.

Efforts towards the preparation of crucial optically pure intermediates for the synthesis of congeners of the naturally occurring polyphosphoinositides will be presented.

Interaction of synthetic D-6-deoxy-myo-inositol 1,4,5-trisphosphate with the Ca^{2+}-releasing D-myo-inositol 1,4,5-trisphosphate receptor, and its effect on 5-phosphatase and 3-kinase metabolic enzymes will be discussed.

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STREPTOZOTOCIN DIABETES REDUCES THE CONTENT OF AN INSULIN-SENSITIVE GLYCOSYL-PHOSPHATIDYLINOSITOL IN ISOLATED RAT HEPATOCYTES

The addition to intact cells of an inositol phospho-oligosaccharide, which is the polar head group of an insulin-sensitive glycosyl-phosphatidylinositol, mimics and may mediate some of the biological effects of this hormone.

Recently, a specific, time- and dose-dependent transport system for this phospho-oligosaccharide has been identified in isolated rat hepatocytes. Here we show that streptozotocin-induced diabetes mellitus reduced (by about 60%) the content of the insulin-sensitive glycosil-phosphatidylinositol in isolated rat hepatocytes. Moreover, streptozotocin-diabetes blocked the hidrolisis of the glycosyl-phosphatidylinositol in response to insulin, diminished hepatocyte uptake of the phospho-oligosaccharide and abolished the stimulatory effect of this compound on glycogen synthesis. Insulin treatment of the animals antagonized the metabolic changes caused by streptozotocin administration. Our results support the hypothesis that insulin resistance in streptozotocin-diabetic rats is related to the impairment of glycosyl-phosphatidylinositol metabolism.

Characterization of a glycosyl-phosphatidylinositol-anchored membrane protein from Trypanosoma cruzi which has crossreactive epitopes with a surface activation antigen of T and B lymphocytes and supresses their activation.

Cristina Hernández-Munaín, Antonio Alcina and Manuel Fresno.

Chagas' disease results from the infection of the protozoan parasite T. cruzi and affects several million people in South América. Four monoclonal antibodies (MAbs) specific for T. cruzi were obtained. Flow cytometry analysis showed that these MAbs stained the membrane of the three main morphological forms of T. cruzi: amastigotes, epimastigotes and trypomastigotes. The four MAbs seemed to recognize the same 50- to 55- KDa antigen that was revealed by immunoblotting. Several experiments indicated that this antigen was a glycosyl-phosphatidylinositol-anchored membrane protein. (i) The antigen could be removed from the cell surface by treatment with proteases, NaOH, HNO2, and phosphatidylinositol-specific phosphoslipase C (PI-PLC). (ii) The phase distribution of the antigen in Triton X-114 solutions changed drastically upon treatment with PI-PLC. The antigen was found mainly in the detergent phase in nontreated samples and in the aqueous phase in PI-PLC-digested samples. (iii) A cross-reacting determinant that was found in other glycosyl-phosphatidylinositol-anchored membrane proteins appeared after PI-PLC treatment.

Several alterations of the immune response have been described during this disease such as severe immunodepression of both cellular and humoral responses, and massive polyclonal stimulation with the generation of autoantibodies crossreacting with host cell and tissues. One of these -50 to -55 KDa antigen-specific MAbs, C10, crossreacts with a 28 KDa antigen expressed on the membrane of >90% of T and B lymphocytes, lypopolysacharide, phorbol dibutyrate ester or antigen and on T and B mouse cell lines. Furthermore, this monoclonal antibody is able to suppress T and B cell proliferation to any of those stimuli. In addition, sera from chagasic patients and T. cruzi-infected mice but not from normal counterparts contain antibodies which recognize a similar antigenic site from in T and B lymphocytes since they specifically inhibit MAb C10 binding to those cells. These results suggest a pathological role of autoantibodies as an alternative mechanism for T. cruzi-associated immunosuppression.

GLYCOSYL PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE D AND ITS ROLE IN MAST CELL SIGNALLING. K.-S. Huang*, W.-J. C. Fung*, P. Lin+, and A. M. Gilfillan+, Departments of Protein Biochemistry* and Pharmacology+, Hoffmann-La Roche Inc., Nutley, NJ 07110

Glycosyl phosphatidylinositol-specific phospholipase D (GPI-PLD) hydrolyzes the inositol phosphate linkage of GPI-anchored proteins and GPI lipids. We have recently purified GPI-PLD from bovine serum, deduced its primary structure, and demonstrated its in vivo activity by co-transfecting the isolated cDNA with a gene encoding a GPI-anchored protein. To further understand the physiological role of this enzyme, we report here its involvement in signal transduction in rat mast (RBL 2H3) cells. Using the Trypanosome variant surface glycoprotein (VSG) as a substrate, GPI-PLD activity was detected in RBL 2H3 cells. When the cells were sensitized with anti-TNP IgE then stimulated with TNP-ovalbumin (TNP-OVA), GPI-PLD activity significantly increased within 15 sec. after stimulation and returned to basal level within 2 min. When the cells were metabolically labelled with [^{3}H]-myristic acid or [^{3}H]-glucosamine and the lipids analyzed by TLC, GPI containing molecules were rapidly lost at 15 sec. after stimulation, but restored to the original levels after 2 min. The time event for the loss of GPI containing molecules was also mirrored by the accumulation of phosphatidic acid, one of the hydrolysis products generated by the action of GPI-PLD. When the cells were preincubated with neutralizing anti-GPI-PLD antibodies for 5 min. followed by stimulation with TNP-OVA, the release of histamine from the cells was completely blocked. These results suggest that GPI-PLD was activated upon stimulation of RBL cells, generating two possible second messengers, a phosphatidic acid and an inositol glycan. Both of these messengers may play a role in signalling histamine release.

REGULATION OF INTERLEUKIN 2 DEPENDENT GROWTH RESPONSES BY GLYCOSYL-PHOSPHATIDYLINOSITOL MOLECULES

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The molecular mechanism of signal transduction through the IL2 receptor remains an enigma. Glycophosphatidyl-inositol (GPI) lipids have been investigated as one component of this process. IL-2 stimulated the rapid loss of >50% of GPI in the IL2 dependent T-cell line CTLL-2. Half-maximal GPI loss was detected at 40 pM IL-2, coincident with the EC_{50} (20 pM) for IL-2-induced proliferation of this cell line. This effect was specifically inhibited by antibodies that bind either IL-2 or the IL-2 receptor. The biological relevance of GPI hidrolysis was demonstrated by the sinergism of purified IPG with IL-2 in T cell proliferation responses. The inclusion of IPG (0.1 uM) in determinations of IL-2 dependent CTLL-2 growth shifted the EC_{50} from 20 to 7 pM IL-2. IPG had no effect on either the number or affinity of IL-2 receptors; therefore, half-maximal CTLL-2 proliferation was obtained at <10% IL-2 receptor occupancy. These results demonstrate that GPI lipids are an important component of the biological response to IL-2.

AN INOSITOL PHOSPHATE GLYCAN DERIVED FROM A TRYPANOSOMA BRUCEI GLYCOSYL-PHOSPHATIDILINOSITOL INHIBITS HEPATIC GLUCONEOGENESIS.

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We have proposed that an inositol phosphoglycan (IPG) is generated in target cells exposed to insulin, due to the hydrolysis of a glycosyl-phosphatidylinositol (GPI) precursor. The IPG reproduces the actions of insulin in both cellular and subcellular assays. The insulin-sensitive GPI is structurally similar to a GPI protein anchor found in numerous cell types and species. Since it is difficult to purify sufficient quantities of IPG, we have prepared a pronase fragment (PF) from a *Trypanosoma brucei* variant surface glycoprotein GPI anchor. The resulting PF contains 1,2 cyclns-P-Gln-(Man)3-P-EtN-ASP. PF produced a dose-dependent increase in lipogenesis in rat adipocytes. The maximal response was approximately 50% of that observed with insulin, and was maximal at approximately 200 µM. Additionally, PF produced a greater than 90% inhibition of isopreterenol-stimulated lipolysis, with maximal effects observed at 70 µM. Among intracellular enzymes examined, hepatic glucose-6-phosphatase was most sensitive. PF caused almost complete inhibition of glucose-6-phosphatase at 80 µM. The glycan reduced the V_{max} with no effect on k_m. Inositol, inositol-2- monophosphate, inositol 1,2 cyclic monophosphate, glucosamine, mannose, mannose-6-phosphate, phosphorylethanolamine, ethanolamine and aspartic acid had no effect on this enzyme. Chemical and enzymatic modifications of PF have revealed details about structure/ activity, leading to a more precise definition of the structural components necessary for the insulin-mimetic actions of inositol glycans.

Insulin- and Glucose-Dependent Release of Lipid-Modified cAMP-Binding Proteins from Plasma Membranes and Mitochondria of the Yeast Saccharomyces Cerevisiae

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The yeast Saccharomyces cerevisiae harbors two cAMP-binding proteins (M_r=54,000 and 46,000) which are anchored to the outer faces of the plasma membrane and the inner membrane of mitochondria, respectively, by covalently attached phosphatidylinositol (PI) moieties (1). The anchor structure of the plasma membrane glycoprotein is characterized by the presence and specific arrangement of the constituents of typical glycosylphosphatidylinositol (GPI)-modified ectoproteins of higher eucaryotes (Müller and Bandlow, in the press). In contrast, the PI moiety of the mitochondrial counterpart lacks additional carbohydrate residues. This was revealed by metabolic labeling and chemical or enzymic digestion of the cAMP-binding proteins and subsequent thin layer chromatography (TLC) analysis of the cleavage products of the anchor structures. The two lipid-modified cAMP-binding proteins are genetically unrelated to the regulatory subunit of the cytoplasmic protein kinase A (Müller and Bandlow, submitted).

In higher eucaryotic cells (e.g. rat adipocytes) hormone (e.g. insulin)-stimlated lipolytic release of GPI-anchored plasma membrane proteins has been observed (2) indicating the existence of a hormone-stimulated GPI-specific phospholipase. It has been speculated that lipolytic and/or proteolytic cleavage products of GPI membrane anchors may act as chemical mediators for the insulin and several other signal transduction cascades. The recently described surprising metabolic and growth effects of insulin on lower eucaryotes (3) suggest the existence of a signal transduction pathway in these organisms which is stimulated by insulin-like molecules. Therefore we studied whether insulin causes cleavage of the lipid membrane anchors of the plasma membrane and mitochondrial cAMP-binding proteins in Saccharomyces cerevisiae.

Yeast spheroplasts grown in lactate medium in the presence of either [^{14}C]stearic acid, [^{14}C]myo-inositol or [^{14}C]ethanolamine

were photoaffinity-labeled with 8-N_3-[^{3}H]cAMP to specifically label the lipid-modified cAMP-binding protein of the plasma membrane (pulse period). Subsequently the cells were incubated in glucose medium (with an excess of the corresponding unlabeled constituent of the lipid anchor) in the presence or absence of human insulin for various times (chase period). The removal of the GPI moiety was monitored by TX-114 partitioning and binding to phenylsepharose of the [^{3}H]labeled protein moiety of cAMP receptor. Insulin caused the concentration-dependent (ED_{50}=50 nM) conversion of the amphiphilic form of the cAMP receptor into the hydrophilic version which partitioned into the aqueous phase during TX-114 phase separation and was unable to bind to phenyl-sepharose. This reaction proceeded rapidly within 15 min with an efficiency of approximately 50 to 60% and strictly required the presence of 0.5 mM D-glucose. Incubation of the cells with glucose alone (in the absence of insulin) also led to the generation of even a greater portion of hydrophilic cAMP receptor protein (up to 80 %) but with a significantly delayed kinetics (2 to 3 h). The possible lipolytic or proteolytic removal of the GPI membrane anchor was assessed by following the retention or loss of the diverse radio-labeled constituents of the GPI moiety used in the pulse/chase experiments. Early after the insulin stimulus a hydrophilic version of the cAMP-binding protein could be identified which had lost the fatty acid label but still harbored the radioactive myo-inositol and ethanolamine residues. With prolonged chase periods this intermediate was converted into a version lacking the inositol and ethanolamine residues. Its apparent molecular weight of 54,000 on SDS-PAGE was identical with the original membrane-bound form (in the absence of insulin).

The data suggest an insulin-dependent two-step processing of the GPI-anchor of the plasma membrane cAMP-binding protein. A lipolytic cleavage (via a GPI-specific phospholipase C or D) is followed by a proteolytic cleavage between the ethanolamine residue of the GPI moiety and an amino acid located close to the carboxyl terminus of the protein moiety. As previously observed, release of the cAMP receptor from the plasma membrane *in vitro* does not only require the cleavage of the GPI anchor by phospholipase C from *Bacillus thuringiensis* but, in addition, the removal of the protein-bound carbohydrate side chain(s) by N-glycanase or the inclusion of high salt. In agreement, insulin did not cause solubilization of the cAMP-binding protein, e.g. it remained attached to the plasma membrane after centrifugation of the spheroplasts, possibly fulfilling its physiological function at the level of the membrane and not in the periplasmic space.

The release of the mitochondrial cAMP-binding protein from the inner membrane into the intermembrane space after addition of insulin and glucose to lactate-grown yeast spheroplasts was studied by photoaffinity-labeling with 8-N_3-[^{3}H]cAMP of isolated mitochondria, pre-labeled with [^{14}C]stearic acid (the binding of cAMP is not affected by the lipid modification). We observed time- and concentration-dependent conversion of the PI-modified amphiphilic cAMP-binding protein into its hydrophilic soluble derivative. This was accompanied by loss of the fatty acid label which suggests the existence of an insulin-dependent phospho-lipase inside the mitochondria and of a signal transduction

cascade between the plasma membrane and mitochondria.

The role of the cAMP-binding proteins of the plasma membrane and mitochondria from the yeast Saccharomyces cerevisiae as well as of the regulated removal of their (G)PI membrane anchor and the possible function of the cleavage products generated thereby remains to be elucidated.

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Urinary Excretion of *Chiro*-Inositol is Linked to Insulin Resistance In Obese Rhesus Monkeys.

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One putative mediator of insulin action has been found to contain D-*chiro*-inositol. An alteration in the metabolism of this mediator may contribute to the insulin resistance associated with type 2 diabetes. We have observed reduced urinary *chiro*-inositol excretion in type 2 diabetic rhesus monkeys and type 2 diabetic humans (NEJM 323:373, 1990). The purpose of this study was to determine if low *chiro*-inositol levels in the urine were associated with insulin resistance in obese prediabetic rhesus monkeys.

A hyperinsulinemic euglycemic clamp and an intravenous glucose tolerance test were performed on 12 middle-aged spontaneously obese (>20% body fat) monkeys distributed across the following groups: obese insulin-sensitive (n=3) (IS), obese insulin-resistant (n=5) (IR), and obese type 2 diabetic (n=4) (DM). 24 hour urinary *chiro*-inositol levels were also determined. The mean ± SD glucose disposal rates (M), glucose disappearance rates ($K_{Glucose}$) and 24 hour urinary *chiro*-inositol excretion (CI) (mean ± SE) for the three groups were as follows:

	IS	IR	DM
M (mg/kg•FFM/min)	7.254 ± 2.12	4.690 ± 0.89	2.490 ± 1.14
$K_{Glucose}$	3.10 ± 1.4	3.16 ± 0.7	1.37 ± 0.2
CI (μM/day)	2.1 ± 1.2	1.2 ± 0.5	0.4 ± 0.1

Significant correlations were observed between insulin-mediated glucose disposal rates and 24 hour urinary excretion of *chiro*-inositol ($r = 0.57$; $p < 0.05$) and between glucose disappearance rate and 24 hour urinary excretion of *chiro*-inositol ($r = 0.56$; $p < 0.05$).

From these preliminary data we conclude that the early insulin resistance present in rhesus monkeys prior to the spontaneous development of obesity-associated type 2 diabetes is associated with a decrease in *chiro*-inositol excretion. Further longitudinal studies will be performed to determine if the absence or decreased excretion of this putative mediator of insulin action in the urine is a marker for the development of insulin resistance and type 2 diabetes in rhesus monkeys.

PHOSPHATIDYLINOSITOL GLYCANS AND PHOSPHATIDYLINOSITOL GLYCAN-ANCHORED PROTEINS IN TETRAHYMENA MIMBRES

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Phosphatidylinositol (PI) glycans of *Tetrahymena* have been characterized by biosynthetic labeling with [3H]myristate, [3H]inositol, [14C]glucosamine, [3H]mannose, and [3H]ethanolamine. The PI glycans were extractable with chloroform:methanol:water (10:10:3), and sensitive to bacterial PI-specific phospholipase C (PLC) and nitrous acid deamination. Chemical analyses of the PI glycans indicated the presence of fatty acids, alkylglyceryl ethers, phosphate, inositol, glucosamine, mannose, and ethanolamine. The main lipid component appears to be sn-1-alkyl, 2-lyso-PI with some sn-1-acyl, 2-lyso-PI. After further purification by TLC followed by chemical analyses and gas chromatography, the PI glycans indicated a pool of glycans composed of PI glycans without ethanolamine (TLC Rf: 0.3-0.6) and with ethanolamine (TLC Rf: 0.1-0.2). The PI glycans migrated on SDS-PAGE with an apparent MW of 10-14 kDa during the initial 12 h following the various radioisotope additions. Subcellular localization over the first 2-12 h indicated the labeled PI glycans were distributed throughout the cell, with the largest proportion at 12 h being associated with a fraction containing mitochondria, lysosomes, and peroxisomes. However, by 24 h the free PI glycan radioctivity had incorporated mainly into a protein with a broad band of 24-29 kDa in a process almost totally inhibited by cycloheximide or tunicamycin. The exact mobility of the protein band within this molecular weight range was dependent on the growth temperature of the cells. Treatment of 24 h-labeled intact cells with the PI-PLC *in vivo* released the labeled protein from the cell. Some labeled proteins were present even in the medium of non-PLC-treated cells. *Tetrahymena* PI glycans appear to accumulate in a metabolic pool from which they are gradually removed for attachment to externally-oriented PI-anchored proteins. *Tetrahymena* is a well suited system for studying the regulation of PI-anchored protein biochemistry.

INSULIN DOES NOT INDUCES THE HYDROLYSIS OF A GLYCOSYL-PHOSPHATIDYLINOSITOL (GPI) IN RAT FETAL HEPATOCYTES. EVIDENCE IN THESE CELLS OF INSULIN RESISTANCE AT POST-RECEPTOR LEVEL.

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An inositol phospho-glycan (IPG) which is the polar head group of a glycosyl-phosphatidylinositol (GPI) has been considered as a possible mediator of insulin action. In an attempt to gain insight into the functions of this hormone during development, the relationships between insulin, insulin receptors, GPI and IPG were studied. GPI was isolated and characterized in fetal liver as soon as day 15 of intrauterine life. In isolated hepatocytes from fetal and adult rats labelled with [^{3}H]-glucosamine, [^{3}H]-galactose or [^{3}H]-myoinositol, these molecules were incorporated into GPI. In hepatocytes labelled with [^{3}H]-glucosamine and then allowed to react with [1-^{14}C]-IAI, the [^{3}H]-labelled GPI was purified as the [^{14}C]-labelled amidinate lipid. GPI molecules from fetal and adult cells were sensitive to hydrolysis by a PI-specific phospholipase C from B. cereus. The product of this hydrolysis,

inhibits the activity of a cAMP-dependent protein kinase, while this effect was abolished by nitrous acid deamination. In isolated hepatocytes from adult animals an inverse correlation between extracellular insulin and the number of insulin receptors and the cellular content of GPI was observed. By contrast, in fetal hepatocytes insulin failed to reduce the GPI content when labelled either with [^{14}C]-IAI or [^{3}H]-glucosamine. Fetal and adult hepatocytes were incubated with insulin or IPG after which glycogen phosphorylase activities were determined. IPG mimicked the action of insulin on both forms of the enzyme from adult hepatocytes, while in fetal cells insulin did not change and IPG reduced the activities of glycogen phosphorylase. These findings suggest a dissociation between insulin receptor occupancy and the expected hormonal effects in fetal hepatocytes. This could represent a state of insulin resistance at a post-receptor level.

Agonist-specific Ca^{2+} oscillations in single rat hepatocytes

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Ca^{2+}-mobilising agonists, which are mediated by inositol 1,4,5-trisphosphate (IP_3), induce free Ca^{2+} oscillations (spikes) in single rat hepatocytes microinjected with aequorin, a Ca^{2+}-sensitive photoprotein. While the frequency of oscillations depends on agonist dose, the time-course of an individual spike does not change with agonist concentration. The time-course of individuals spikes does, however, depend on the agonist species. While the rise time and amplitude do not change with the agonist, the falling time, and hence the spike duration, is markedly dependent upon the type of the agonist, in the same individual hepatocyte (1). Elevated cAMP increases the frequency and peak free Ca^{2+} of spikes induced by phenylephrine (Phe) (2). In contrast, when the agonist is vasopressin (Vp) elevated cAMP prolongs the falling phase of the transients. Moreover, ryanodine, which blocks the Ca^{2+}-induced Ca^{2+} release (CICR) channel in the open conformation, has almost no effect on Phe-induced oscillations (1); in contrast ryanodine does inhibit Vp-induced spikes. In certain circunstancies ryanodine can truncate the tail of Vp-induced spikes. So, we propose that Vp spikes consist of an IP_3-mediated symmetrical spike, similar in time-course to those induced by Phe, followed by a "tail" that represents CICR. We explain the ability of ryanodine to completely abolish Vp-induced spikes by envisaging that the CICR-releasable pool of Ca^{2+} is supplied solelyfrom the IP_3-releasable pool; translocation of Ca^{2+} from the one to the other is promoted by Vp but not by Phe. It is conceivable that Vp receptor activity can modulate Ca^{2+} pooling within a cell. The agonist-specifity of the effect of cAMP could be explained by an enhancent of Ca^{2+} accumulation by the IP_3-sensitive pool. Thus, in the presence of Phe a faster efflux from the IP_3-sensitive pool would be predicted (raising peak free Ca^{2+}), whereas Vp spikes would assume a prolonged tail because the additional luminal Ca^{2+} had translocated from the IP_3-sensitive pool to the CICR-releasable compartment.

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Phospholipid "Second Messengers" in Insulin Action in BC3H-1 Myocytes : Roles of Diacylglycerol, G-Proteins and a PI-Specific Phospholipase C. ML STANDAERT, M del C VILA, G MILLIGAN, DR COOPER, RV FARESE. Tampa, FL and Glasgow, Scotland.

Physiological concentrations of insulin (INS) regulate phospholipid metabolism in BC3H-1 myocytes in a time and dose-dependent fashion by multiple mechanisms including the *de novo* synthesis of diacylglycerol (DAG) as well as through the hydrolysis of phosphatidylcholine and phosphatidylinositolglycan. These pathways generate multiple "second messengers" which mediate the effects of INS in differentiated myocytes. Rapid INS-induced increases in DAG are associated with 2-3 fold increases in glucose transport while the generation of a phospholipid-derived low-molecular weight (< 5 kDa) cytosolic factor is associated with a 2-fold increase in glycerol-3-phosphate acyltransferase (G3PAT) activity.

INS stimulation of hexose transport was mimicked in a non-additive fashion by treatment with biologically active phorbol esters and was blocked by inhibitors of protein kinase C (PKC). Treatment with a PI-specific phospholipase C (PI-PLC) did not stimulate glucose uptake. In contrast, INS activation of G3PAT was not mimicked by phorbol esters, was not blocked by PKC inhibitors, but was stimulated in a non-additive fashion with insulin by treatment with a PI-PLC. INS stimulated a 2-fold increase in microsomal G3PAT through a decrease in Km with no change the Vmax. INS activation of G3PAT was pertussis toxin-sensitive and in cell-free preparations INS activation of G3PAT was blocked by treatment with NaF, a phosphatase inhibitor, as well as by treatment with either an antiserum raised against a PI-specific PLC (gift of Dr. J. Fox) or an antiserum that recognizes the C-terminal decapeptide of $Gi\alpha$. Antisera recognizing $Go\alpha$ and $Gs\alpha$ were ineffective.

In conclusion, these results suggest that dual phospholipid-derived "second messengers" : diacylglycerol and a low-molecular weight cytosolic factor, respectively, mediate INS effects on glucose transport and GP3PAT activity in BC3H-1 myocytes via bifurcating pathways. However, these pathways appear to be closely integrated since activation of G3PAT may account for INS-induced increases in the synthesis of DAG. This, in turn, may amplify both DAG-PKC signalling of hexose transport and serve as a mechanism to replenish inositolglycan containing phospholipid reservoirs which are hydrolyzed during insulin action.

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ROLE OF GLYCOSYL-PHOSPHATIDYLINOSITOL
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240 **Genetic Strategies in Development.**
Symposium in honour of Antonio García Bellido. Lectures by S. Ochoa, S. Brenner, G. S. Stent, E. B. Lewis, D. S. Hogness, E. H. Davidson, J. B. Gurdon and F. Jacob.

244 **Course on Genome Evolution.**
Organized by E. Viñuelas. Lectures by R. F. Doolittle, A. M. Weiner/N. Maizels, G. A. Dover, J. A. Lake, J. E. Walker, J. J. Beintema, A. J. Gibbs, W. M. Fitch, P. Palese, G. Bernardi and J. M. Lowenstein.

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

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

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

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

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

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

253 **Workshop on Genome Expression and Pathogenesis of Plant RNA Viruses.**
Organized by F. García-Arenal and P. Palukaitis. Lectures by D. Baulcome, R. N. Beachy, G. Boccardo, J. Bol, G. Bruening,

J. Burgyan, J. R. Díaz Ruiz, W. G. Dougherty, F. García-Arenal, W. L. Gerlach, A. L. Haenni, E. M. J. Jaspars, D. L. Nuss, P. Palukaitis, Y. Watanabe and M. Zaitlin.

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

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

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

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

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

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

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Organized by N. Sonenberg and L. Carrasco. Lectures by V. Agol, R. Bablanian, L. Carrasco , M. J. Clemens, E. Ehrenfeld, D. Etchison, R. F. Garry, J. W. B. Hershey, A. G. Hovanessian, R. J. Jackson, M. G. Katze, M. B. Mathews, W. C. Merrick, D. J. Rowlands, P. Sarnow, R. J. Schneider, A. J. Shatkin, N. Sonenberg, H. O. Voorma and E. Wimmer.

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Organized by M. Perucho and E. Martínez-Salas. Lectures by D. Gelfand, K. Hayashi, H. H. Kazazian, E. Martínez-Salas, M. Mc Clelland, K. B. Mullis, C. Oste, M. Perucho and J. Sninsky.

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

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Organized by T. A. Springer and F. Sánchez-Madrid. Lectures by S. J. Burakoff, A. L. Corbi-López, C. Figdor, B. Furie, J. C. Gutiérrez-Ramos, A. Hamann, N. Hogg,

L. Lasky, R. R. Lobb, J. A. López de Castro, B. Malissen, P. Moingeon, K. Okumura, J. C. Paulson, F. Sánchez-Madrid, S. Shaw, T. A. Springer, T. F. Tedder and A. F. Williams.

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