Regulation of Cell Growth

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Imaging biological material with scanning probe microscopes

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Our interest for the use of scanning-probe microscopes (scanning-tunneling (STM) and atomic-force (AFM)) in biology derives from the theoretical possibility of observing living cells at high resolution. We have chosen to first visualize surfaces of increasing levels of difficulty and then to study biological membranes and intracellular organelles. We first developed a special technique to observe the same portion of a specimen by STM, scanning electronmicroscopy and light microscopy. Atomic, respectively molecular resolution could be easily achieved with the Nanoscope® for inorganic (graphite) and organic (anthracene-crystals). For biological specimens we lightly fixed the material with OsO4 (for STM) and paraformaldehyde (for AFM) and mapped the surface of cultured cells and erythrocytes with an 80x80 µm scan-head. Structural components in the cytoplasm of muscle fibers were visualized *in air or in buffer after* sectioning with a cryo-ultramicrotome. The morphology observed with the scanning-probe microscopes is conform to that seen with other types of microscopes. At the moment the resolution is comparable to that seen with electronmicroscopy.

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ADULT HEART CELLS UNDER METABOLIC STRESS SHOW IN CULTURE MITOCHONDRIA WITH INCLUSIONS ENRICHED FOR CREATINE KINASE

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Adult rat cardiomyocytes (ARC) in culture undergo extensive changes in morphology and in expression of contractile proteins similar to that found in fetal heart cells. In contrast to fetal cells, though, ARC accumulate mitochondrial creatine kinase (Mi-CK) and show, by using antibodies against this protein, two types of mitochondria: large, cylindrically shaped randomly distributed mitochondria exhibiting a strong signal for Mi-CK, and small round mitochondria organized in rows parallel to myofibrils and giving a much weaker signal. EM investigations revealed in the large mitochondria paracrystalline intracristal inclusions which were specifically decorated by gold-coupled Mi-CK antibodies. Both, large mitochondria and their inclusions *disappear upon addition* of 20 mM creatine to the creatine-free culture medium. We suggest that a low intracellular total creatine level (creatine plus phosphocreatine) causes a metabolic stress and the high concentration of Mi-CK within the inclusions may represent a compensatory effect.

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LOCALIZATION OF ACTIVATED AND NON-ACTIVATED FIBRINOGEN RECEPTORS ON SPREAD PLATELETS USING THE IMMUNOGOLD TECHNIQUE

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The glycoprotein IIb-IIIa complex (GP IIb-IIIa) on the surface of stimulated platelets is the receptor for fibrinogen and mediates platelet aggregation. Although GP IIb-IIIa is present on the surface of unstimulated platelets, platelet activation is required to form the binding site for fibrinogen.
We have prepared two monoclonal antibodies (MAbs): pl-62 binds to the inactive and active form of GPIIb-IIIa, whereas the MAb pl-55 only recognizes the functionally active form. The immunogold-technique was used to identify and localize the two forms of GP IIb-IIIa on the surface of spread platelets. Gel-filtered platelets were allowed to adhere for 3 min on collodium/carbon-coated Ni-grids. After mild fixation, the platelets were incubated with either pl-62 or pl-55 and the bound MAbs were detected with a gold-labeled anti-mouse IgG antibody. On a fully spread platelet the total amount of GP IIb-IIIa, as identified by pl-62, was always 3 to 5 fold higher than the number of active receptors as recognized by the MAb pl-55. Occasionally, GP IIb-IIIa formed clusters in the plane of the membrane, which contained active as well as inactive receptors. We conclude that GP IIb-IIIa exists in two molecular forms on fully spread platelets.

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IMMUNOCYTOCHEMICAL LOCALIZATION OF AN ANTIGEN POSSIBLY INVOLVED IN ACETYLCHOLINE RELEASE.

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A monoclonal antibody (8/38A) raised against cholinergic synaptosomal plasma membranes isolated from *Torpedo* electric organ, was found to inhibit the Ca^{+2}-dependent release of acetylcholine induced in *Torpedo* synaptosomes.

By indirect immunolabeling of semi-thin sections of the electric organ, the antibody labeled specifically a thin and continuous band corresponding to the synaptic region.

To localize the antigen at the ultrastructural level we used gold-immunolabeling of cryoultrathin sections. Results showed clearly that the antibody 8/38A was recognizing an antigen located on the membrane of the nerve terminals. As seen by double-labeling, it codistributed with acetylcholinesterase, a known presynaptic marker in this tissue.

Using label-fracture techniques, we could observe that the antibody 8/38A was binding to the surface of synaptosomes isolated from *Torpedo* electric organ.

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VISUALIZATION OF THE SPATIAL RELATIONSHIP BETWEEN GLIA AND VASCULARIZATION WITHIN THE VERTEBRATE RETINA BY CONFOCAL SCANNING OPTICAL MICROSCOPY

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The euangiotic mammalian retina contains two glial cell types, namely Müller cells (radial glia) and astrocytes. Although differences in their functions are not established yet, they can be distinguished by a few morphological criteria such as cell shape, distribution, and orientation within the retina. The only region where cytoplasmic processes of astrocytes and Müller cell intermingle is the optic nerve fiber layer with its numerous blood vessels. In an attempt to elucidate the spatial relationship between glia and vascularization, we have performed double immunocytochemical staining of retinal whole mounts. Astrocytes were labelled for glial fibrillary acidic protein (GFAP), Müller cells for vimentin, and vessels for collagen IV or for smooth muscle α-actin. By confocal scanning optic microscopy, we describe the pattern of ensheathment of blood vessels by the two glial cell types. By serial optic sectioning and computer-directed reconstruction, this technique allows to follow within the tissue, single cytoplasmic processes over extended distances.

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IMMUNOSTAINING, A NEW VERY SENSITIVE TECHNIQUE TO LOCALIZE DNA IN THIN SECTIONS

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A new immunostain-technique is introduced to reveal DNA on thin sections of cryofixed and freeze-substituted specimens which were embedded in Lowicryl HM20. The essential feature of the method consists of an amplification procedure: The surface of thin sections is first reacted with a mouse IgM specific for double stranded DNA (Boehringer: lot No. 100 03 399). In the second step the IgM is coated with a specific goat IgG against the IgM. The deposited large amount of proteins on the surface of the section is now stained with a mixture (1:2) of aqueous solutions of 2% KMnO4 and 2% uranylacetate. The resulting contrast is so strong that identification of DNA containing areas is very easy. With this technique we could show details of the distribution of chromatin within nuclei of Euglena gracilis where the heterochromatin is predominantly stained. Even in mitochondria and chloroplasts DNA could be detected. Because this highly sensitive technique detects even very small areas of DNA, the nucleoid of E. coli was shown to be highly dispersed ("coralline").

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MEMBRANE SKELETON OF THE CILIATE PSEUDOMICROTHORAX DUBIUS: AN IMMUNOLOGICAL APPROACH TO STUDY THE SPATIAL DISTRIBUTION OF ITS PROTEINS

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The membrane skeleton of P. dubius is a protein layer called epiplasm surrounding the whole cell, just beneath the plasma and alveolar membranes. By scanning electron microscopy this layer includes some particularly differenciated structures, as the ciliary rows and, between them, transverse epiplasmic ridges. This epiplasm is composed of many proteins, some of which are glycosylated. For instance 62, 53, 48 and 36 kDa polypeptides are labeled with Concanavaline A, and are strongly cross-reactive with each other when using polyclonal antibodies. These glycoproteins were extracted from the total epiplasm fraction and used to raise monoclonal antibodies. These antibodies were screened on western-blots and then by fluorescent and gold-labeling to determine localization within epiplasm. While most clones established so far label many bands on western-blots and label the entire epiplasm, by fluorescence and electron microscopy, a few clones have a more restricted labeling pattern for specific regions of the epiplasm membrane skeleton.

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THE FRACTAL DIMENSION OF LYMPHOCYTES AND LEUKEMIC CELLS

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EM image profiles of normal blood lymphocytes and of neoplastic lymphoid cells obtained from humans with acute lymphoblastic leukemia, classified according to surface marker phenotype, were recorded by digital image processing and computer devices and the fractal dimension D evaluated with the probabilistic method of the moments. Normal T lymphocytes had a D quite similar among five moments ,i.e., from 1.21 to 1.23. Cells of an Hairy-cell leukemia with highly convoluted morphology displayed a higher D comprised between 1.32 and 1.36, whereas blast cells of T and B lymphoblastic leukemia were characterized by a smaller D ranging from 1.10 to 1.11. Interestingly, when normal T lymphocytes were transformed into blasts by in vitro stimulation with PHA, the fractal dimension D was found quite close (1.11-1.12) to the latter. In conclusion,lymphoid blasts generated in vitro or of neoplastic origin are immature proliferating cells with less irregular profiles than control lymphocytes. This smoothness might be related to the more fluid configuration of lipids in the plasma membrane of blasts.

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CELLS TRANSFECTED WITH CLONED MICROTUBULE-ASSOCIATED PROTEIN 2 DNA UNDERGO CYTOSKELETAL REARRANGEMENT

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The neuron-specific microtubule-associated protein MAP2 is believed to influence neuronal morphogenesis by promoting the formation of microtubules and cross-linking them. The low mol. wt. form of MAP2, MAP2c, is of particular interest because it is expressed only in the developing brain. To find out more about its function we cloned cDNA for rat brain MAP2c into a eukaryotic expression vector and transfected it into a variety of non-neuronal cell lines that do not normally express MAP2. In all cell types tested the exogenous MAP2c was completely bound to microtubules. In the kidney epithelial cell line PLC, the expressed MAP2c induced bundling of the microtubules, which are normally separately from one another. It also caused a striking rearrangement of the microtubules, so that instead of emanating singly from a centrally located organizing centre they occured as thick bands, located peripherally in the cell and frequently forming rings under the cell membrane. This powerful influence of MAP2c on the microtubular cytoskeleton clearly illustrates the potential of this one molecule for organizing the internal structure of the axons and dendrites in which it occurs.

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CATHEPSIN B AND ITS INHIBITOR CYSTATIN C : CO-LOCALIZATION, CO-EXPRESSION AND CO-SECRETION IN HUMAN COLON CARCINOMAS.

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Cathepsin B activity has been found in tissues extracts and ascites from tumors including colon carcinomas. It has also been correlated with the invasive/metastatic potential. The cystatins are natural inhibitors of this cysteine proteinase. Using mono- and polyclonal antibodies against cathepsin B and cystatin C on semi-serial sections from human colon carcinoma tissues, both antigens appeared by immunofluorescence to be associated with tumor cords and to have a heterogeneous distribution. Cathepsin B activity was found in conditioned media of 5 colonic cell lines mainly as a latent form activable by pepsin treatment. Cystatin C antigen was also recovered in these conditioned media demonstrating a co-secretion of both antagonists. Northern blot analysis confirmed this co-expression in all cell lines (2.3 and 4.3 kb for cathepsin B, 0.8 kb for cystatin C). A recombinant cystatin C, E64 and diazomethane-cysteine proteinase inhibitors, were used to inhibit successfully the total secreted activity of the proteolytic enzyme. Inhibitors are used in invasion and degradation assays to define the precise involvement of the regulated Cathepsin B / Cystatin C system in invasive events.

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IN VITRO MIGRATION OF *HYDRA* NEMATOCYTES ON THE NATURAL EXTRACELLULAR MATRIX, ON TYPE IV COLLAGEN AND ON LAMININ

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Nematocytes (stinging cells) of the fresh water coelenterate *Hydra* are thought to at least partly use the mesoglea, the extracellular matrix, as a substratum for their ameboid migration from the body column of the polyps, where they differentiate, to the tentacles where they are used mainly for prey capture and defense.

We have developed a system to study the behavior of isolated nematocytes in vitro. Using this in vitro-system and video microscopic techniques, we have determined the migration speed and migration frequency of nematocytes on the mesoglea and on cover slips coated with type IV collagen or laminin (which are two major components of the extracellular matrix). Cells migrated randomly on all tested substrata. The average migration speed on mesoglea was about 370µm/h, on type IV collagen about 420µm/h and on laminin about 50µm/h. Whereas nematocytes migrated in a fairly continuous manner on the mesoglea and on laminin, cell movement on collagen was interrupted by periods of stagnation. The maximal migration speed on collagen was found to be 800µm/h.

The determined migration parameters were correlated with the cell morphology in a SEM-study and with the patterns of actin and tubulin by immunocytochemical methods.

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THE ATTACHMENT OF *HYDRA* NEMATOCYTES TO FIBRONECTIN, TO TYPE IV COLLAGEN AND TO LAMININ

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Fibronectin, type IV collagen and laminin are components of the mesoglea, the extracellular matrix of the fresh water coelenterate *Hydra*. Nematocytes (stinging cells) are thought to at least partly use the mesoglea as a substratum during their ameboid migration from the body column of the polyps to the tentacles Nematocytes differentiate in the body column from a stem cell population (the interstitial cells) and are used in the tentacles mainly for prey capture and defense.

In order to eventually dissect the molecular mechanisms of nematocyte migration, we have analyzed the attachment of nematocytes to artificial surfaces coated with type IV collagen, with laminin and with fibronectin. On fibronectin we have determined the degree of attachment in dependence of the concentration. In addition we have compared the cell morphology as it appears in scanning electron microscopy and the pattern of the major cytoskeletal proteins actin and tubulin determined by immunocytochemical methods in nematocytes attached to the different substrata.

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PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST ISOLATED CENTROSOMES BY IN VITRO IMMUNIZATION OF MURINE SPLEEN CELLS.

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Centrosomes contain the centrioles consisting mainly of tubulin. The components of the pericentriolar material are not well characterized. Centrosomes can be isolated from cells cultured in suspension (Bornens et al.: Cell Mot. Cyt. 8, 238-249 (1987)) or from calf thymus tissue (Komesli et al.: J. Cell Biol. 109, 2869-2878 (1989)). Methods were adapted that allowed the preparation of 5 liter of cell suspension per working cycle yielding 10^9 centrosomes or 25 µg protein per preparation. 10^9 centrosomes were solubilized in formic acid and coupled to 7 nm silica beads by absorption. Following a modification of the Vitrotech (BioInvent, Lund) immunization protocol the silica/antigen complex was incubated with $2.1x10^8$ spleen cells in the presence of $5x10^6$ peritoneal macrophages. After fusion many hybridoma cell lines were obtained and the produced antibodies were screened by immuno-fluorescence. Over 50% of 70 selected hybridoma clones showed a vimentin like staining pattern. Several monoclonal antibodies were characterized, which localized in the cell in the centrosomal region.

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3D-Visualization of Biological Structures Using Confocal Microscopy

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Confocal microscopy has become an important tool for biologists to investigate the 3D morphology in a variety of samples. In combination with objectives of high numerical aperture the confocal arrangement allows 3D localization of structures. Recording series of 2D images (xy-plane) along the axial direction (z-axis) leads to a complete volume representation of the sample. There is a large number of algorithms that are summarized by the term "3D-reconstruction". They accept 3D raster or object data as input and produce one or a series of images suitable for interpretation by the human visual system. Two types of algorithms are presented: 1) the 3D raster data are first interpreted to produce a list of graphical objects which are then visualized using computer graphics. 2) the algorithm uses the raw raster data introducing light reflections, light scattering and shadows. The latter technique is referred to as "ray tracing" and leaves the interpretation to the human eye. With these procedures tissue preparations as well as the intracellular organization of single cultured cells were studied.

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SURFACE MORPHOLOGY OF VITRIFIED ULTRATHIN SECTIONS

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Electron microscopy of vitrified ultrathin sections allows the study of cell ultrastructure in the hydrated state. Sectioning of the frozen material, however, causes severe mechanical deformation such as longitudinal compression in the cutting direction and reorganisation of the section surface into deformation lines parallel to the knife edge. On stereo views of micrographs and by geometrical argumentations we find that those deformation lines affect only one surface. Except for knife marks, the opposed section surface is smooth. The observations can be interpretated by the following cutting model: The rough surface develops on the former block face of the specimen from material that is squeezed out of the section plane when the section is compressed in the cutting direction and bent away from the specimen block. The other section surface is kept smooth by its contact with the knife whereby also the knife marks are imprinted.

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NUCLEAR CYTOPLASMIC TRANSPORT: A NEW *IN VITRO* SYSTEM REVEALS REGULATION BY A CASEIN KINASE II SITE

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A new *in vitro* system for nuclear cytoplasmic transport was established and used to analyse the regulation of nuclear protein transport by phosphorylation. HTC cells were mechanically perforated according to Simons & Virta [EMBO J. **6** (1990) 2241] and transport was measured by quantitative laser fluorescence microscopy. The passive permeability of the nuclear envelope was as *in vivo* (functional pore radius ~5.0 nm). Specific protein transport required cytosolic extract and ATP. As *in vivo* the transport of large recombinant proteins containing the nuclear localization sequence (NLS) of the SV40 T-antigen [Rihs & Peters, EMBO J. **8** (1989) 1479] was much enhanced by a NLS-flanking sequence. Site-directed mutagenesis showed the casein kinase II (CK-II) site (T-antigen residues 111/112) to be responsible for this. The CK-II inhibitor heparin abolished the transport enhancement. Residues 111/112 were phosphorylated by incubation with cytosol *in vitro* .

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IMMUNOCYTOCHEMICAL DOUBLE-LABELLING ON PANCREAS THIN SECTIONS – QUESTIONING THE ZYMOGEN GRANULES' HOMOGENEITY

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For a very long time reports have supported the view of a morphologically and functionally homogeneous exocrine pancreas. Only for the last ten years reports argueing this view have emerged and ever more studies support the hypothesis of pancreatic heterogeneity. Specifically, more than one population of zymogen granules differing in their contents have been proposed which release their zymogens differentially according to changing digestive demands. Electron microscopy techniques do not only allow insight in morphology, but in combination with immunocytochemistry may provide detailed information on antigen presence and distribution. We have performed indirect double-labelling experiments on Lowicryl K4M embedded pancreatic tissue of rats, using antibodies directed against (pro)lipase, trypsin(ogen) and phospholipase A2. We expected to see differences in relative labelling densities of the three respective antigens and we have indeed found absolute and relative variations of the labelling density per zymogen granule unit area. Our results strongly support the idea that more than one population of zymogen granules coexist in the pancreatic acinar cells of the rat.

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THE ROLE OF THE TRANS GOLGI NETWORK (TGN), COATED VESICLES, VESICLE FUSION AND CONDENSATION, AND PROPROTEIN PROCESSING IN SECRETORY GRANULE FORMATION

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Secretory granules (trichocysts) of a ciliated protozoan consist of an e^--translucent, paracrystalline core ("shaft") bordered on each of its 4 sides by an e^--dense rod ("arm"). Shaft and arm material originate from clear vesicles (100-160 nm Ø) and dense vesicles (40-80 nm Ø), respectively, which form in the TGN. Clear vesicles lose their coat rapidly and fuse with the dense, coated vesicles to form the early presecretory granule. The latter, which can be labeled by trichocyst-specific Ab's, enlarges either by fusion with clear, uncoated vesicles and dense, coated vesicles, or by fusion with other, larger presecretory granules. When the granule attains ~ 2 µm Ø, the e^--translucent core condenses to form the paracrystalline shaft and the e^--dense granules form the DBA-positive arms. The role of condensation, crystallization and proprotein proteolysis in presecretory granule maturation is presented based upon comparison of secretory and nonsecretory cell lines, and Selenium-induced proprotein proteolytic processing.

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IMMUNOCYTOCHEMICAL LOCALIZATION OF THE GOLGI APPARATUS USING PROTEIN-EPITOPE PURIFIED ANTIBODIES TO GALACTOSYLTRANSFERASE

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The main subcellular site of glycan biosynthesis is the Golgi apparatus (GA); thus, polyclonal antibodies to galactosyltransferase (GT) have been widely used as immunohistochemical reagent for the GA: However, a significant portion of the antibodies were shown to be glycan-specific. To unequivocally prove peptide-specificity of the antiserum used and to ascertain the merit of using GT antibodies as GA marker, recombinant GT was expressed in E. coli as a fusion protein with ß-galactosidase (r-GT). r-GT crossreacted by immunoblotting with polyclonal antisera to human milk GT and thus was used as an immunosorbent to purify protein-epitope specific polyclonal antibodies. These antibodies stained the GA in HeLa cells and CaCo2 cells.

To learn more about the immunodominant peptide portion of GT, rGT was truncated to the C-terminal part presumably containing the active site. Weak crossreactivity with polyclonal antisera was detected by immunoblotting, while no staining of the GA was observed. Moreover, a polyclonal rabbit antiserum against the truncated rGT fusion protein reacted stongly with ß-galactosidase but did not bind to milk GT. Thus, we conclude that the stem region of the enzyme contains the immunodominant peptide portion.

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SECRETORY ORGANELLES IN TWO PROTOZOAN CELLS: IMMUNOLOGICAL CHARACTERIZATION OF THEIR MAJOR PROTEINS.

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Trichocysts are extrusive organelles found in Ciliate cells like *Pseudomicrothorax dubius* and *Paramecium tetraurelia*. Separation of *P. dubius* trichocyst proteins shows more than 20 bands on SDS-PAGE and more than 40 spots on 2-D gels after Coomassie staining. Polyclonal antibodies were raised against the Con A-negative 26 and 27Kd bands. On immunoblots, anti-26/27Kd serum recognizes 20-25 spots with pI's between 6.3 and 4.6. Some spots are only recognized by α-(26/27Kd), whereas others are also recognized by two polyclonal antibodies, produced against either the 30/31Kd or the 15-20Kd bands. The anti-30/31Kd serum labels 15-20 spots with pI's between 5.8 and 4.6. The anti-15/20Kd serum labels 25-30 spots with pI's between 6.6 and 4.6. By EM after immunogold labeling, α-(26/27Kd) specifically labels the trichocyst shaft of *P. dubius*, but never its four trichocyst arms, suggesting there is no immunological homology between shaft and arms. Trichocyst homologies are revealed between *P. dubius* and *P. tetraurelia* after immunolabeling on 2-D Western blots and on ultrathin sections.

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POSSIBLE SIGNAL TRANSDUCTION THROUGH THE B1 INTEGRIN, VLA-4.

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Monoclonal antibody MX24 which recognizes a heterodimeric structure of 135-145 KDa has recently been identified as an antibody specific for the α4 chain of the β1 integrin, VLA-4. This was shown by immunodepletion experiment using a reference anti-VLA-4 mAb (kindly provided by Dr M. Hemler) whereby in detergent solubilized lysates of ^{125}I-labeled CEM cells, after depletion with mAb MX24, reference anti-VLA-4 was no more able to precipitate the two polypeptide chains of 135 and 145 KDa characteristic for VLA-4. In contrast to other anti-VLA-4 mAbs, functional studies showed that MX24 induced high levels of IL-2 production in Jurkat cells. In addition incubation of these cells with MX24 resulted in Ca^{2+} mobilization from internal stores. In peripheral blood mAb MX24 was found to react with 39-76% of resting T cells in individual donors. Two-color flow microfluorimetry showed that the MX24 positive cells were equally distributed among the $CD4^+$ and $CD8^+$ subset. Incubation of peripheral blood T cells with mAb MX24 resulted in IL-2 production and cell proliferation. In addition, anti-VLA mAb MX24 induced homotypic adhesion of cells from the myelomonocytic cell line U937. Moreover, cell adhesion triggered through VLA-4 could not be inhibited by preincubation of the cells with an anti-LFA-1α mAb, 6B11.

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ADHESION MOLECULES EXPRESSION AND REGULATION ON NEUROBLASTOMA CELLS AND SUBCLONES.

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The expression of adhesion molecules involved in cellular adhesion, and immune recognition has been measured on a neuroblastoma cell line SK-N-SH and 3 phenotypically different subclones, SY5Y, SH-EP and SH-IN. A great variation in the pattern of expression of adhesion molecules was measured by flow cytometry on the 4 clones. Whereas the highly malignant neuronal SY5Y cells were characterized by low HLA-ABC, ICAM-1, LFA-3 and high N-CAM expression, the less malignant epithelial-like SH-EP clone expressed moderate to high levels of HLA-ABC, ICAM-1, LFA-3, VLA-2, but low levels of N-CAM molecules. Cytokines and differentiation inducers treatment of the clones revealed that HLA-ABC, ICAM-1 and LFA-3 were variably regulated by rTNFα, IL-1 and rIFNγ and differentiation inducers. In addition, cytokine-mediated enhancement of adhesion molecules expression was accompanied by an increase of sensitivity of NB clones to LAK lysis.

The parallel between the pattern of expression of adhesion molecules and tumorigenic phenotype of the clones as well as the increase of their expression upon differentiation, suggest that these molecules might participate in mechanisms of immune recognition and growth control.

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ADAPTIVE RESPONSE OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES (PBL's) TO LOW DOSES OF IONIZING RADIATION.

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An altered frequency of chromosome aberrations in human PBL's has been reported, by various investigators, if a given challenge dose (1.5 Gy x-rays) is preceeded by a low adaptive dose (0.01 Gy x-rays). This phenomenon can not be explained simply as the sum of the effects of the two doses. In order to examine cell cycle dependence of the "adaptive response" we studied chromosome aberration yields after challenge doses given either in G1-, S- or G2-phase. The adaptive dose was given in the G1-phase. A protective adaptive response was found in none of the samples from two donors. If the challenge dose was given in G2, a significant increase in aberration yields was observed.

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MULTI-LEVEL REGULATION OF THY-1 ANTIGEN EXPRESSION IN MURINE T LYMPHOMA

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Thy-1, a murine T cell marker probably involved in transmembrane signalling, is attached to the cell surface via a glycosylphosphatidyl-inositol (GPI) anchor. To define the possible mechanisms regulating its expression, we have analysed T lymphoma lines for Thy-1 cell surface and gene expression. It was previously shown, that in one lymphoma variant line ($S1A^{-b}$), Thy-1 is not attached to the cell via the GPI anchor but released in the medium. We show that Thy-1 release is not due to an alteration in the GPI anchoring signal sequence encoded by Thy-1 mRNA, confirming that the biosynthetic pathway of the GPI anchor is affected in the $S1A^{-b}$ line. This Thy-1 negative mutant line shows also an 8-fold decrease in the amount of Thy-1 mRNA influencing only slightly the total amount of Thy-1 polypeptide. This reduction in Thy-1 mRNA steady state is mediated at the level of transcription. Complementation of both defects,e.g. in the GPI anchoring biosynthetic pathway and in Thy-1 gene expression, can be achieved in somatic cell hybrids indicating that a specific trans-enhancing mechanism increases the basal level of Thy-1 gene expression in T cells. Our data suggest that regulation Thy-1 antigen occurs not only at the post-translational level but also at the transcriptional and translational level.

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HLA CLASS II GENOTYPING BY HYBRIDIZATION WITH OLIGONUCLEOTIDES IMPROVES THE SELECTION OF UNRELATED DONORS FOR BONE MARROW TRANSPLANTATION

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Histocompatibility typing allows the matching of patients and donors in organ transplantation and the accuracy of HLA matching influences the clinical outcome. Molecular studies of HLA class II genes revealed the existence of a very large degree of allelic polymorphism. In view of the limitations of current serology we have developped a direct genotyping procedure which consists in the hybridization of PCR-amplified DNA with sequence-specific oligo probes (oligotyping). The analysis of HLA-DRB1 (42 alleles), DRB3 (3 alleles), DRB5 (4 alleles), DQB1 (14 alleles) and DPB1 (19 alleles) by oligotyping showed differences at one or several loci among serologically-matched individuals. The technique is predictive of a positive mixed lymphocyte culture and can therefore improve HLA class II typing and the selection of an optimally matched donor for bone marrow transplantation.

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CONTROL OF IL-2 RECEPTOR LIGHT CHAIN EXPRESSION BY INTERLEUKIN-1, TUMOR NECROSIS FACTOR AND INTERLEUKIN-2: COMPLEX REGULATION VIA ELEMENTS IN THE 5'FLANKING REGION.

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We have analysed the mechanisms by which interleukin 1 (IL1), interleukin 2 (IL2) and tumor necrosis factor (TNF) regulate expression of IL2 receptor α chains (IL2Rα) in a rodent T cell line. All three cytokines induce detectable IL2Rα mRNA by themselves, but there is strong synergy between IL1 or TNF, on one hand, and IL2, on the other. The earliest phase of induction by IL1 is independent of protein synthesis. IL1, but not TNF, also stimulates transient secretion of IL2. This leads to an autocrine stimulation of a further increase in IL2Rα mRNA levels. When IL2 secretion has dropped off, continued IL2Rα expression requires both IL2 and IL1. Most or all of this regulation is due to changes in the rate of transcription of the IL2Rα gene. It depends on a segment spanning from -733 to -2500 in the IL2Rα 5'flanking region, upstream of all cis-acting regulatory elements previously identified in the human gene. We are now in the process of identifying the cytokine responsive elements by introducing deletions and checking these constructs in vivo in transient transfection assays.

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IN VITRO SECRETION OF IgM IN RESPONSE TO STAPHYLOCOCCAL PROTEIN A (SPA) DEPENDS ON CONTAMINANT ENTEROTOXINS AND SECRETION OF IgM RHEUMATOID FACTORS (RF) DEPENDS ON THE SPA Fcγ-BINDING ABILITY

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SPA, considered as a polyclonal B cell activator, interacts with the Fc portion of human IgG and could play a role in differentiation of B cells in RF secreting cells.

Using an ELISPOT technique, we investigated the role of SPA on IgM and IgM RF production, in cultures of human blood mononuclear cells.

SPA increased the number of IgM secreting cells but not recombinant SPA. Staphylococcal enterotoxins, present in natural SPA but absent in recombinant SPA, seems to be the cause of this difference.

SPA induced more IgM RF ELISPOTS than PWM when compared to total IgM production. Induction of RF seems dependent of SPA Fcγ-binding ability as inactivation of the four tyrosil residues, essential for its Fcγ-binding, abolished the specific stimulation of RF secreting cells.

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ROLE OF PROTEIN KINASE C (PKC) IN EICOSANOID SYNTHESIS IN MOUSE MACROPHAGES

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Mouse peritoneal macrophages responded to zymosan stimulation by rapid enhancement of arachidonic acid (AA) release and subsequent PGE2 and LTC4 synthesis. Similarly, combinations of phorbol ester PMA and calcium ionophore synergistically induced eicosanoid formation. Staurosporine, a potent inhibitor of PKC, completely abolished zymosan-induced PGE2 and LTC4 generation. Furthermore, down-regulation of PKC strongly attenuated zymosan-stimulated AA release and eicosanoid synthesis. Immunoblot experiments with PKC isozyme-specific antibodies demonstrated that PKC-β is the predominant isoform present in macrophages. PMA stimulation caused translocation and subsequent down-regulation of PKC-β. These data suggest that PKC plays a critical role in the regulation of zymosan-induced AA release and eicosanoid synthesis. The contribution of PKC isozymes to eicosanoid synthesis is under investigation.

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SITE OF MALATE SYNTHASE SYNTHESIS (GERMINATING SOYBEAN)

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Isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4.1.3.2) are the two specific enzymes of the glyoxylate cycle. This pathway connects lipid catabolism and gluconeogenesis in plant cells, and occurs in glyoxysomes.
The oligomeric structure of malate synthase (dimers, octamers or superaggregates) depends on ionic strength (subunit Mr ~ 64'000) [1], and two hypotheses have been formulated regarding the synthesis site of the enzyme (free polysomes or endoplasmic reticulum [2,3]).
Organelles fractions from 2-day germinated soybean cotyledons were obtained and characterized using sucrose gradients, marker enzymes and ELISA tests (reticulum/mitochondria/glyoxysomes). Superimposition of enzyme activity determinations and ELISA tests results provides the means to evaluate the validity of the above mentioned hypotheses.

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EXPRESSION OF CATALYTICALLY ACTIVE RAT 5-ALPHA-REDUCTASE IN *SACCHAROMYCES CEREVISIAE*.

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5-alpha-reductase is an integral membrane protein which converts testosterone to dihydrotestosterone (DHT). DHT is the principal androgen responsible for growth of the human prostate. In adult males, DHT has been implicated in the development of benign prostatic hyperplasia. Very little is known about the physical properties of 5-alpha-reductase since the enzyme has never been purified. To better characterize the enzyme, we have expressed catalytically active rat 5-alpha-reductase in *Saccharomyces cerevisiae*. A cDNA encoding rat 5-alpha-reductase was fixed in frame to the signal sequence of yeast acid phosphatase. The cDNA was then ligated into a yeast expression vector downstream of the acid phosphatase promoter. *S. cerevisiae* transformed with the 5-alpha-reductase expression plasmid produced a functionally active enzyme which rapidly converted testosterone to DHT. Thus, the yeast expression system can be used to produce large amounts of the membrane bound 5-alpha-reductase in an enzymatically active form.

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IONIC CHANGES DURING CELL-CELL FUSION OF SFV INFECTED AEDES ALBOPICTUS CELLS.

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Semliki Forest Virus infected Aedes albopictus cells exhibit syncytium formation upon exposure to mildly acidic pH. The mechanism of the process leading to membrane fusion is as yet unknown. The occurrance of a membrane hyperpolarization as well as transmembrane fluxes of protons, K^+ and Na^+ have already been described (Kempf et al., Biosci. Rep. 8 (3), 241-253, 1988). We now investigated the minimal conditions required for the membrane hyperpolarisation to take place. The presence of chloride is necessary to obtain a maximal hyperpolarization. Furthermore, the hyperpolarization is inhibited by the anion-transport-inhibitor DIDS. It seems therefore, that the change in membrane potential is mediated through changements in anion permeability. Chloride flux in Aedes cells was measured by using the Cl^- sensitive fluorescent indicator MQAE. Under fusogenic conditions, Aedes cells exhibit an influx of chloride. Concomitantly, the intracellular Calcium level is slightly elevated.

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THE GENES FOR THE HOMOLOGOUS CA^{2+}-BINDING PROTEINS ONCOMODULIN AND PARVALBUMIN ARE LOCATED ON DIFFERENT HUMAN CHROMOSOMES

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Parvalbumin (PV) and oncomodulin (OM) are structurally homologous Ca^{2+}-binding proteins. Whereas OM is an oncodevelopmental and tumor specific protein, PV is present in fast skeletal muscles and GABA-ergic neurons of vertebrates. In addition to the high similarity of the coding sequences, intron/exon structures of the genes encoding these high-affinity Ca^{2+}-binding proteins are identical. This supports the hypothesis of a common ancestor for these genes. The aim of this study was to investigate whether these genes are clustered in the human genome. The OM gene was found to be located on human chromosome 7 using the human-hamster cell hybrid technique. No linkage was found with the PV gene, sublocalized on human chromosome 22 q12-q13 by the same technique using cell lines containing different distinct fragments of human chromosome 22. In addition, gene dosage analysis of human DNA with chromosome 22 supernumeracy showed that the PV gene is located distal from the locus of the Cat Eye Syndrome (CES), characterized by multiple congenital malformations.

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STRUCTURE OF A 100 KB HUMAN GENE CODING FOR THE PLASMA MEMBRANE CALCIUM ATPASE ISOFORM 1

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Plasma membrane Ca^{2+} ATPases (PMCAs) are key enzymes responsible for the specific transmembrane transport of Ca^{2+} against large concentration gradients. In the human genome, a multigene family consisting of at least four members encodes multiple PMCA isoforms. The structure of the first human PMCA gene (hPMCA1) has now been elucidated. It contains a minimum of 22 exons spread over more than 100 kb of DNA. Exon 1 contains exclusively 5' untranslated sequences; the protein-coding region is specified by exons 2 to 22. However, at least 3 out of the 21 protein-coding exons may or may not be included in the mature mRNA due to various pathways of alternative RNA splicing, and additional, as yet undefined, non-constitutive exons may also be present. The sequence of the putative promoter region indicates that this member of the PMCA gene family may be of the "housekeeping" type. Although the number of exons in the hPMCA1 gene is similar to that in the gene for a rabbit SR Ca^{2+} pump, the sizes of the two genes are vastly different and the positions of intron interruptions with respect to the protein-coding sequence are not well conserved between these two Ca^{2+} pump genes. SNSF 3.531-0.86, 31-27103.89, 31-28772.90.

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MODULATION OF ALDOSTERONE SYNTHESIS BY Ca^{2+} AND Mg^{2+} IN ISOLATED ADRENAL MITOCHONDRIA

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Angiotensin II and K^+, two physiological aldosterone stimulators, raise cytosolic Ca^{2+} in adrenal glomerulosa cells. In permeabilised cells, Ca^{2+} entry into mitochondria stimulates aldosterone production. In order to locate the site of Ca^{2+} action, we investigated its effect on the late steps of aldosterone synthesis in mitochondria from bovine adrenal zona glomerulosa. Steroids produced by coupled mitochondria with either 11-deoxycorticosterone (DOC) or corticosterone (B) as precursors, were analysed by HPLC and aldosterone was measured by RIA. In the absence of Mg^{2+}, low concentrations of extramitochondrial Ca^{2+} inhibited by 73±7% (N=12) the conversion of DOC or B to aldosterone. Conversion of DOC to B was inhibited by 71% by Ca^{2+}. This effect was dose-dependent (EC_{50}:300nM), and was abolished by ruthenium red (1 μM) and Mg^{2+}, two mitochondrial Ca^{2+} uniporter inhibitors. Mg^{2+} prevented Ca^{2+} inhibition dose-dependently with a maximal effect at 1 mM.

In summary, Ca^{2+} inhibits the conversion of both DOC to B, and B to aldosterone, steps wich are believed to be mediated by the same enzyme. Physiological $[Mg^{2+}]$ prevents the Ca^{2+} induced inhibition of aldosterone synthesis from DOC. We conclude that Ca^{2+} stimulation must occur at an earlier step, possibly on the cholesterol side chain cleavage.

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HORMONE-INDUCED $[Ca^{2+}]_i$ OSCILLATIONS IN HEPATOCYTES: MECHANISM AND MODULATION IN THEORY AND PRACTICE

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The frequency encoding of hormone signals via hormone-induced $[Ca^{2+}]_i$ oscillations in hepatocytes can be explained by interaction of calmodulin with $InsP_3$-receptor activated calcium channels of intracellular calcium stores. The rate laws of our associated theoretical model have a mathematical structure reminiscent of the Brusselator. We experimentally demonstrated that phenylephrine-induced calcium oscillations, as observed in single heptocytes, can be inhibited by calmodulin antagonists (calmidazolium, CGS9343B). In cells overstimulated by phenylephrine, as characterized by a non-oscillatory elevated $[Ca^{2+}]_i$, these antagonists could again restore sustained $[Ca^{2+}]_i$ oscillations. Similar results were seen with agents elevating cAMP (forskolin, 8CPTcAMP), which act analogously to an increase of hormone, and protein kinase C activators (dioctanoyl glycerol, RG80267), which inhibit the oscillations. Such experimental observations, including the modulation of the oscillations by extracellular calcium, were predicted by our mathematical model. (SNSF grant no. 3.042-087)

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CA^{++} AND THE REGULATION OF C-FOS GENE EXPRESSION IN THE PROMYELOCYTIC CELL LINE HL-60

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Various Ca^{2+} agonists including hormones, neurotransmitters, depolarizing agents and Ca^{2+} ionophores are known to cause rapid induction of the proto-oncogene c-*fos*. It is however uncertain whether Ca^{2+} modulates c-*fos* gene expression directly or indirectly via production of other signals. It is noteworthy in this respect that in most studies high Ca^{2+} agonist concentrations that do not selectively raise cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) were used and $[Ca^{2+}]_i$ was not measured. To gain insight into the action of Ca^{2+} in this process, we determined the $[Ca^{2+}]_i$ dose dependence of c-*fos* induction in promyelocytic HL-60 cells differentiated with DMSO. Cells were incubated with the Ca^{2+} ionophore ionomycin in the absence of external Ca^{2+} or in the presence of increasing external Ca^{2+}. $[Ca^{2+}]_i$ was monitored using fura^2 under the same conditions where the level of the c-*fos* transcript was measured. The threshold of c-*fos* induction was ~120-200 nM and a maximal approximately 40 fold induction occurred at ~400 nM Ca^{2+}. Surprisingly, higher Ca^{2+} (0.6-1 uM) caused substantially less transcript accumulation. It is concluded that the c-*fos* gene is exquisitely sensitive to Ca^{2+}, responding to Ca^{2+} in the very low submicromoler range. At high but still physiological Ca^{2+}, the effect of the ion is much less pronounced. The mechanism underlying this phenomenon remains to be explored.

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Ca^{2+} Modulation of Redox State in Single Pancreatic ß-cells.
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An increase of reduced pyridine nucleotide (NAD(P)H) fluorescence precede Ca^{2+} influx in nutrient stimulated single ß-cells. We examined whether intracellular ionized calcium ($[Ca^{2+}]_i$) elevation, a prerequisite for insulin secretion, modulates, in turn, the redox state in the ß-cell. Single rat islet ß-cells were studied by fluorescence microscopy at the appropriate wavelengths for (NAD(P)H) and fura-2. Insulin secretion from the perfused rat pancreas following similar protocols was monitored to validate the single cell observations.
At 8.3 mM glucose, $[Ca^{2+}]_i$ and NAD(P)H oscillations occurred with similar frequency. Then, carbachol or tolbutamide, a blocker of ATP-sensitive K^+ channels were used to test whether an imposed rise in $[Ca^{2+}]_i$ influences the redox state in the ß-cell. When Tolbutamide or carbachol were applied at stimulatory glucose concentrations, in the presence of 2-ketoisocaproate or pyruvate, a further increase in NAD(P)H fluorescence followed the $[Ca^{2+}]_i$ elevation. However, at non-stimulatory glucose, tolbutamide but not carbachol increased transiently NAD(P)H fluorescence. Furthermore, carbachol and tolbutamide were weak secretagogues when added at low glucose to the perfused pancreas, but synergized with nutrients to promote insulin secretion. Thus, the nutrient dependent alterations in redox state by Ca^{2+}, reflect the metabolic adjustments of the activated ß-cell, allowing the continuous generation of coupling factors to sustain or potentiate insulin secretion.

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EXTRACELLULAR ATP STIMULATES INSULIN SECRETION BY Ca^{2+} DEPENDENT AND INDEPENDENT MECHANISMS
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The mechanism by which extracellular ATP stimulates insulin secretion was examined in RINm5F cells. ATP raised cytosolic Ca^{2+} ($[Ca^{2+}]_i$) and depolarized the cells (EC_{50}=5 uM) as measured with fura-2 and bisoxonol respectively. The $[Ca^{2+}]_i$ rise encompasses Ca^{2+} mobilization and Ca^{2+} influx. The former component and inositol trisphosphate generation were inhibited by phorbol-myristate-acetate (PMA) which uncouples agonist receptors from phospholipase C. This manoeuvre did not block Ca^{2+} influx or membrane depolarization. Diazoxide (opening ATP-sensitive K^+ channels) attenuated membrane depolarization and part of the ATP-evoked Ca^{2+} influx which was not affected by L-type channel blockers. ATP increased the rate of insulin secretion by more than 12-fold. Prolonged exposure to EGTA dissociated the $[Ca^{2+}]_i$ rise from ATP-induced insulin secretion, since the former was abolished and the latter only decreased by about 60%. PMA only reduced ATP-induced insulin secretion by 34%. These results suggest that ATP stimulates insulin release by both Ca^{2+} dependent and independent mechanisms. The latter points to a direct triggering of exocytosis by ATP.

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INTRACELLULAR CALCIUM POOLS IN DIFFERENTIATING PC12 CELLS
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The rat clonal pheochromocytoma cell line (PC12) contains caffeine- and IP_3-sensitive intracellular Ca^{2+} stores. We studied changes in the free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) that are related to calcium release from these stores before and after one week of NGF-induced differentiation. Changes in $[Ca^{2+}]_i$ were recorded by means of fura-2 single cell microfluorimetry. Basal $[Ca^{2+}]_i$ of cells at rest (70-80 nM) was not altered by long-term treatment with NGF, neither in the cell bodies nor in the growth cones. Addition of 30 mM caffeine caused a fast transient rise in $[Ca^{2+}]_i$ in cell bodies (720±75 nM; n=10). A much smaller increase in $[Ca^{2+}]_i$ (280±40 nM; n=9) could be observed in 9 out of 14 growth cones; the others did not respond. The extent of filling of the caffeine-sensitive pool affected basal $[Ca^{2+}]_i$. The Ca^{2+} storage sites, which were empty under normal culture conditions, were filled by a single K^+-depolarization, followed by spontaneous depletion (50% in about 5 min) after washout of high $[K^+]_o$. Bradykinin-induced increase in $[Ca^{2+}]_i$ (404 ±60 nM; n=6) due to a release from the IP_3-sensitive pool was independent of the state of filling of the caffeine-sensitive pool and was only detected in cell bodies. We conclude that the two pools are not interconnected and are of minor importance in growth cones. The spatial dynamics of the Ca^{2+} signal due to influx through voltage-gated or ATP-activated channels were different from those caused by release from intracellular stores.

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Differential regulation of cytokine expression in human fibroblasts (WI38) by dexamethasone (dexa)
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Tumor necrosis factor alpha (TNF) rapidly increases expression of several cytokines such as GM-CSF, M-CSF, IL-6 and NAP-1 at both the protein and RNA levels. Dexa markedly reduces TNF-mediated stimulation of GM-CSF, IL-6 and NAP-1 expression at both the protein and RNA levels. In contrast, dexa does not suppress M-CSF expression. We realized that M-CSF has no AU-rich sequences in the 3' untranslated region as opposed to GM-CSF, IL-6 and NAP-1 suggesting that one possible pathway of the dexa-mediated suppression might be via these AU-rich sequences. Current work is directed towards elucidating possible mechanisms of this differential regulation by dexa, e.g. by transfection experiments with constructs where globin cDNA is linked to the AU-rich sequence of GM-CSF or to an artificial GC rich sequence.

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The Porcine Tumor Necrosis Factor Genes: Genomic Sequence and Comparative Analysis
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We have cloned and sequenced a 10.22 kbp fragment of the porcine genomic tumor necrosis factor (TNF) locus. A partially digested liver DNA library was cloned into lambda phage EMBL4 and screening was performed with a porcine TNF-α cDNA probe. Analysis showed that both the TNF-α and the TNF-ß genes were present in a tandem arrangement as has been observed in other species. In addition to the two genes, the insert contained about 2 kbp of medium and highly repetitive sequences at its 5' end. Some of these repeats display features of matrix attachment regions suggesting that they may be of importance for the regulation of the TNF genes. Comparison of the regions flanking the genes, exons and introns was performed against human, mouse and rabbit TNF genes. Several binding sites for transcription factors in the TNF gene promoters were discovered during this analysis. The sequence data will enable us to study the regulation of TNF genes in context of porcine viral diseases.

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EPIDERMAL GROWTH FACTOR RECEPTOR: ITS POSSIBLE IMPLICATION IN THE MAINTENANCE OF HEPATOCYTE MASS IN BILIARY CIRRHOSIS
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Epidermal growth factor stimulates DNA synthesis; changes of the number and properties of its receptor (EGFR) occur during regeneration and may be of importance in the maintenance of hepatocellular mass in liver cirrhosis. We therefore studied the changes in EGFR (distribution+number) in the development of cirrhosis induced by bile duct ligation in rats (BDL,n=21), sham operated animals serving as controls (CTR,n=17). 3-28 days after BDL, liver lobes were fixed in paraformaldehyde-glutaraldehyde for immunohistochemistry using a monoclonal antibody against EGFR. The cellular distribution of EGFR in CTR showed a strong labelling of cytoplasm (88±SD4% positive) and plasma membranes (89±18%) but no labelling of nuclei. In BDL in contrast, cytoplasmic staining was decreased already after 3 days of BDL (15±9%) and the labelling of canalicular membranes (15±7 vs 0% in CTR) and nuclei (27±26%) was increased after 14 days of BDL. The shift of EGFR from plasma membrane and cytoplasma to nuclei supports the idea that EGFR is involved in the maintenance of hepatocyte mass in this model of cirrhosis.

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CHARACTERIZATION OF THE HIGH AFFINITY NERVE GROWTH FACTOR RECEPTOR ON RAT PC12 CELLS

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PC12 cells have been reported to express high and low affinity receptors for nerve growth factor (NGF). Only one gene has been identified which codes for both the high and the low affinity NGF-receptor. Two hypotheses have been brought forward to explain interconversion between low and high affinity receptors:
a) the high affinity receptor represents the internalized receptor,
b) the high affinity receptor represents a complex between the low affinity receptor and an unidentified protein.
To distinguish between the two hypotheses we determined the two receptor subtypes under conditions where receptor internalization is inhibited, i.e. low temperature and in cell lysate. Computer-assisted non-linear least square analysis of equilibrium binding at 4°C clearly showed the existence of two binding sites. Similarly whole cell lysates showed two binding sites. Furthermore, kinetic analysis of receptor ligand binding at 4°C clearly showed the existence of high affinity binding. The results indicate that the high affinity receptor is plasma membrane-located and pre-exists in the absence of NGF.

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HIGH CELL DENSITY RECOMBINANT *E.COLI* FERMENTATION

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A recombinant W3110 *E.Coli* strain expressing a fusion of the OmpA signal sequence and the "Human Granulocyte-Macrophage Colony Stimulating Factor" (hGMCSF) was fermented using a high cell density process. Growth was monitored by several measurements: optical density, carbon dioxide evolution, glycerol fed, residual glycerol and acetate production. Optical density and dissolved oxygen were used as the basis for controlling the feed rate of glycerol (sole carbon source). hGMCSF expression and solubility were determined by PAGE and Western Blot Analysis. Biological activity assays gave an estimate of the amount of mature hGMCSF. We also investigated the processing of the fusion protein into hGMCSF and its translocation from the cytoplasm into the periplasmic space.

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IN VITRO MODELS FOR STUDYING TISSUE REPAIR. THE EFFECTS OF A HAEMODIALYSATE CLINICALLY USED TO IMPROVE WOUND HEALING

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In vivo processes involved in wound healing, such as inflammation, chemotaxis, cell proliferation, cell differentiation and remodelling of the extracellular matrix (ECM) are interdependent. In vitro assays were developed to study influences on isolated processes involved in tissue repair. The effects of Solcoseryl, a low molecular weight haemodialysate from calf blood, were tested.
During granulation tissue formation fibroblasts migrate into the wound space. The influence of Solcoseryl on fibroblast migration was investigated on wounded monolayers of confluent fibroblasts.
Blood supply is essential for cell proliferation and tissue regeneration. B14F28 hamster fibroblasts are usually cultivated in 10% FCS. Proliferation, i.e. ^{3}H-Thymidin uptake of B14F28 cells grown without serum in absence and presence of Solcoseryl was compared.
Fibroblasts grown within 3D collagen lattices undergo processes comparable to ECM remodelling in vivo. This "dermal" equivalent was used to study the influence of Solcoseryl on the process of wound contraction.

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PRODUCTION OF TRANSFORMING GROWTH FACTOR-β IN ASTROCYTES AND GLIOBLASTOMA CELLS

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In inflammatory diseases of the central nervous system the fate of infiltrating T- and B cells may not only depend on presentation of antigen by glial cells but also on local production of cytokines that regulate the immune response. An inhibitor of T cell growth was found to be produced by human glioblastoma cells and belongs to the family of the transforming growth factors-β (TGF-β). This factor, named TGF-β2, was isolated and characterized. In the present study, we report that not only transformed glial cells but also astrocytes express TGF-β2 protein but not TGF-β1. In order to determine the regulation of gene expression of TGF-β in the brain, we cloned and sequenced the promoter region of TGF-β2. A comparison with the TGF-β1 promoter indicates that they differ considerably. The TGF-β2 promoter contains three putative TATA boxes which are lacking in TGF-β1. A very common regulatory element, AP-1, is present in 4 copies in the TGF-β1 promoter but not in TGF-β2. The AP-1 DNA binding protein is a heterodimer of jun and fos. Therefore glioblastoma and neuroblastoma cells were investigated for concomitant mRNA expression of c-jun, c-fos and TGF-β1.

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THE MACROPHAGE RESPONSE IN INFLAMMATORY AND NEOPLASTIC TISSUES OF THE BRAIN: GM-CSF PRODUCTION BY ASTROCYTES AND GLIOBLASTOMA CELLS

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Astrocytes and microglial cells may play a central role in regulation of immune mediated processes in the central nervous system. By their inducible expression of MHC class II antigens and secretion of cytokines they may propagate expansion and activation of T and B lymphocytes invading the brain tissue. Here we report that astrocytes may also contribute to the microglia/macrophage response observed in inflammatory, neoplastic and degenerative diseases of the brain. Astrocytes from newborn mice secrete granulocyte-macrophage colony-stimulating factor (GM-CSF) as evidenced by induction of colony formation in bone marrow cells and growth of FDC-P1 cells. Both effects are neutralized with anti-GM-CSF but not with anti-IL-3 antibodies. Some residual activity detected in the bone marrow assay after antibody treatment can be explained by concomitant production of granulocyte CSF (G-CSF). The mRNA of both G- and GM-CSF are identified in astrocytes and the latter in glioblastoma cell lines. However, in vivo GM-CSF can not be detected in tumor cyst fluids or glioblastoma tissues of the patients.

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EXPRESSION AND MODULATION BY γ-INTERFERON OF α-SMOOTH MUSCLE ACTIN IN CULTURED FIBROBLASTS.

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Primary and passaged cultures of dermal fibroblasts contain a sub-population of cells positive after staining with an antibody (anti α-SM1) specific for α-smooth muscle (SM) actin (Skalli et al.,J Cell Biol,103:2787,1986), the actin isoform typical of arterial SM cells. These cells could correspond to contaminating SM cells or pericytes or represent fibroblasts modulated in culture, as in clinical (Sappino et al.,Int J Cancer,41:707,1988) or experimental (Darby et al.,Lab Invest,63:21,1990) situations, where fibroblastic cells acquire more or less permanently morphological and biochemical features of SM cells.

We have systematically investigated by immunofluorescence (IF) the presence of α-SM actin in different cultured fibroblasts and clones obtained by limiting dilution. We have also studied the action of γ-interferon (γ-IFN) on α-SM actin protein and mRNA expression in fibroblasts by IF and Western blot with anti α-SM1 and by Northern blot with actin mRNA specific probes. Our results indicated that α-SM actin appears constantly in a sub-population of fibroblasts irrespective of the source of cells and even after cloning, thus indicating that α-SM actin expression is not due to contaminant SM cells or pericytes but is a feature of fibroblastic cells themselves. This supports the view that fibroblastic cells represent an heterogeneous population. Furthermore, γ-IFN decreased the expression of α-SM actin mRNA and protein and could be a good candidate to exert anti-fibrotic activity *in vivo*.

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INTERFERON-α2a AND FLUOROPYRIMIDINES: *IN VITRO* EFFECTS AS SINGLE AGENTS OR IN COMBINATION

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Interferons exhibit inhibitory effects on a wide variety of human tumor cells both *in vitro* and *in vivo*. We have observed previously a synergistic effect *in vitro* between IFN-α2a and 5'-deoxy-5-fluorouridine (5'-dFUrd) using a liquid proliferation assay (Experientia 46: A60, 1990). A clonogenic assay (Eliason *et al.*, 1985) has been used to confirm our data, and to determine antiproliferative effect of IFN-α2a alone. The synergistic effect observed between IFN-α2a and 5'-dFUrd has indeed been confirmed with this assay system. IFN-α2a used at doses ranging from 100 to 1,000 IU/ml, exerts a cyto-inhibitory effect on colony formation by 6 out 7 colon carcinoma cell lines of our test panel. Our anchorage-independent growth assay system proved to be more sensitive to measure the IFN-α2a cells responsiveness (IC_{50} = 100-500 IU/ml).

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EFFECTS OF CYTOKINES ON HIV-1 REPLICATION IN HUMAN MONOCYTE CULTURES

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We studied the effects of hematopoietic growth factors on the susceptibility of target cell monocyte cultures to infection with HIV isolated from seropositive individuals at various stages of HIV infection. Virus was transmitted to monocyte cultures by cocultivation. Virus donor cells consisted of peripheral blood mononuclear cells of HIV seropositive individuals at CDC stages II, III, and IV. Normal peripheral blood derived monocyte cultures served as target cells and were grown under varying conditions using colony stimulating factors (CSF) as media additives. Media variations included: No additive, GM-CSF, M-CSF, GM- and M-CSF, and some PHA and IL-2. In cocultures from HIV infection stages II and III replication of HIV was strongly affected by added CSF. Preference for one or the other cytokine was different for each individual. In cocultures obtained from individuals in stage IV of HIV infection, virus isolation was in 5 out of 6 cases not affected by cytokines. Virus isolation was successful on all macrophage cultures independent of the growth media used. In later stages of HIV infection virus variants are formed that have acquired the capability to replicate in macrophages of different stages of cellular differentiation.

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STIMULATION OF NERVE GROWTH IN THE INACTIVE NEUROMUSCULAR SYSTEM: SIGNALING BY INSULIN-LIKE GROWTH FACTORS.

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Upon lesion or paralysis processes in the inactive muscle fibers and in nearby cells are initiated to bring about restoration of muscle activity. Among these is the local stimulation of nerve growth.Insulin-like growth factors (IGFs) can induce neurite growth in vitro, and contents of IGFs mRNA in muscle correlate with the presence of nerve growth inducing activity in fast skeletal muscle. We are therefore investigating the possible roles of IGFs in neuromuscular regeneration, including intramuscular nerve sprouting.We found that: 1.)embryonic motoneurons in vitro respond to IGFs in the subnanomolar range with a vigorous neurite growth and ramification reaction; 2.) non-lesioning application of IGFs to adult rodent skeletal muscle in vivo specifically elicits processes that are observed in denervated or paralysed muscle, including a marked nerve sprouting reaction; 3.) paralysis leads to rapid (detectable after 15h) and transient (peak after 48h) induction of IGF1 mRNA in muscle fibers, followed by a slower and persistent increase of IGF1 and IGF2 mRNA in muscle interstitial cells.

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CYTO-INHIBITORY EFFECTS OF TRANSFORMING GROWTH FACTOR BETA ON HUMAN COLORECTAL CANCER CELL LINES.

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We have established 3 new colorectal cancer cell lines (LS411N, LS513 and LS1034). Cell surface antigen expression has been measured by flow cytometry and monoclonal antibodies to carcinoembryonic antigen (CEA). Expression of major human histocompatibility complex antigens (MHC) has been analysed as well. All these 3 cell lines grow well in 5% FCS medium without addition of growth factors and the LS1034 cell line proliferates in serum free medium containing 0,1% BSA supplemented with transferrin and insulin. We have analysed the responsiveness of these cell lines to $TGF\beta_1$ using liquid proliferation and methylcellulose based clonogenic assays. Both LS513 and LS1034 cell lines reveal responsive to the cyto-inhibitory effect of $TGF\beta_1$ at very low concentrations (EC_{50}= 1 ng/ml and 0,03 ng/ml, respectively). Two other colorectal cancer cell lines (HT-29 and LS411N) do not respond to $TGF\beta_1$ either in liquid or in semi-solid medium proliferation assay. The effect of $TGF\beta_1$ is modulated by retinoic acid (RA) and rHu interferon alpha (IFNα): both RA (10^{-6}M) and IFNα (1000 IU/ml) counteract the growth inhibition induced by $TGF\beta_1$ in LS513 cells.

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LOW AFFINITY NERVE GROWTH FACTOR RECEPTOR ON GLIAL CELL LINES

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Receptors for nerve growth factor (NGF) exist in two states: high-affinity (class I) and low-affinity (class II). Biological responses are thought to be mediated by class I receptors, the role of class II receptors is less clear. In order to find a model system to study class II receptors and their role in neural regeneration, we have screened glial cell lines for NGF receptors. Rat Schwannoma subclones RN6, RN22 and RT4-D6P2T from PNS, as well as glioma-derived cell lines 33B (rat) and 86HG-39 (human) from CNS are shown to bind NGF specifically.
NGF equilibrium binding was investigated by non-linear least-square analysis. All glial lines showed exclusively low-affinity binding (K_d in the nanomolar range) with widely varying B_{max} values. Kinetic studies of the NGF binding confirmed the presence of class II receptors only.
In summary, PNS Schwannoma and CNS glioma cell lines are a suitable model to investigate the role of NGF.

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ACETYL COA CARBOXYLASE AS A CANDIDATE KEY REGULATORY ENZYME IN NUTRIENT INDUCED INSULIN RELEASE

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We have proposed that malonyl- and long chain-CoA esters provide a crucial link between nutrient metabolism and insulin secretion (Prentki, M., and Matschinsky, F.M. Physiol. Rev. 67:1185, 1987). The formation of malonyl CoA by acetyl CoA carboxylase (ACC) is the rate-limiting step in lipid biosynthesis. We therefore aimed at gaining insight into the short and long term regulation of this enzyme in insulin secreting cells. The expression of the ACC gene was studied in normal and clonal (HIT) ß-cells. We detected high levels of ACC mRNA in both cell types. Total ACC activity in insulin-secreting cells was close to that of liver, a lipogenic tissue, and was present at substantially higher levels than in other tissues including heart, adrenals, pancreas, lungs, spleen and kidneys. Stimulation of HIT cells with a combination of the nutrient stimuli glucose plus glutamine plus leucine caused a rapid 3-fold increase in the initial ACC activity which coincided with the initiation of insulin secretion. The results demonstrate that ACC is expressed at high levels in insulin-secreting cells and that nutrient secretagogues induce marked activation of the enzyme. This supports the concept that the formation of malonyl CoA esters plays essential role linking fuel metabolism to insulin release.

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LONG CHAIN ACYL COA-ESTERS AS METABOLIC COUPLING FACTORS IN INSULIN SECRETION

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We have proposed a metabolic model in which malonyl-and long chain acyl CoA-esters are coupling factors mediating the link between fuel metabolism and insulin release (Corkey, et al. J. Biol. Chem. 264: 21608, 1989). We tested this hypothesis by evaluating the action of exogenous fatty acids and fatty acid oxidation inhibitors on insulin release and the acyl-CoA content of clonal pancreatic ß-cells (HIT). Both exogenous palmitate (C:16) and myristate (C:14) (2-20 uM free concentration) were potent stimulators of insulin release. The action of the saturated fatty acids was chain length dependent, being half maximal at C:10 and maximal at C:16. The effect was reversible and inhibitable by somatostatin. The non-metabolizable analogue 2Br-palmitate, an inhibitor of fatty acid oxidation, was also a potent secretagogue. The cellular content of long chain fatty acyl CoA-esters rose by 2-3 fold in the presence of exogenous palmitate. Glucose, either in the absence or presence of palmitate did not translocate C-kinase to a membrane compartment, suggesting that the insulinotropic action of glucose and fatty acids is not mediated via C-kinase. The results lend additional support for the hypothesis proposing that acyl CoA esters are metabolic coupling factors in the signal transduction process of nutrient stimuli.

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POSSIBLE INVOLVEMENT OF SMALL GTP-BINDING PTOTEINS IN THE REGULATION OF INSULIN SECRETION

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Previous experiments indicated a regulatory site in insulin secretion sensitive to GTP analogues and located beyond the generation of second messengers. Since *ras*-related small molecular mass GTP-binding proteins (SMGs) have been implicated in the regulation of secretion in yeast, we characterized their homologues in the insulin secreting cell lines RINm5F and HIT-T15. SMGs were detected either by [^{32}P]GTP binding or by using specific antibodies after gel electrophoresis and transfer to nitrocellulose membranes. These proteins have been proposed to cycle between a membrane-bound and soluble form depending on the liganded nucleotide, we therefore analysed their subcellular distribution during stimulation of insulin secretion in intact and permeabilized cells. At least 13 SMGs in the 20-27 kDa range were found, none of which redistributed during stimulation of intact cells. In contrast, stimulation of insulin secretion from permeabilized cells with GTP[S] or GppNHp, increased the amount of the ADP-ribosylation factor (ARF) in a fraction enriched in Golgi and plasma membrane markers. However, when the concentration dependency of ARF redistribution and the stimulation of secretion by GTP[S] and GppNHp were compared, the EC_{50} values did not indicate a direct link between the two processes. The localization of the other SMGs was not influenced by the guanine nucleotides. Thus, the role of SMGs in insulin secretion remains to be established.

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REGULATION OF THE SYNTHESIS OF THE INSULIN SENSITIVE GLUCOSE TRANSPORTER GLUT4 BY CATECHOLAMINES IN BROWN ADIPOSE TISSUE.

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Insulin stimulates glucose transport in rodents brown adipose tissue up to 50 fold. This tissue is also under the control of the sympathetic system. In several physiological or pathophysiological situations, this tissue becomes insulin-resistant. In these same situations, a decreased sympathetic turnover of catecholamines has been reported. The effect of a ß-adrenergic agonist on the basal and insulin-stimulated glucose transport has therefore been measured in control and insulin-resistant rats. In similar situations, GLUT4 mRNA, as well as the amount of GLUT4 protein have been measured. The data show that short term (24 h.) treatment with the ß-adrenergic agonist increases basal and insulin-stimulated glucose transport as well as GLUT4 mRNA and protein, in control and in insulin-resistant rats. This effect is specific for brown adipose tissue and is not observed in other tissues. This increase may be one of the mechanisms by which catecholamines modify basal and insulin stimulated glucose transport in brown adipose tissue. They suggest, in this tissue, a role for catecholamines in the regulation of the number of insulin-sensitive glucose transporters acting on the synthesis and/or the stability of GLUT4 mRNA.

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RAT ADIPOSE TISSUE UP-REGULATION OF LIPOPROTEIN LIPASE mRNA IN HYPERINSULINEMIC STATES.

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Lipoprotein lipase (LPL) is responsible for the hydrolysis of VLDL/chylomichron triglycerides and plays a significant role in adipose tissue lipid accumulation. In animal models of obesity, LPL activity has been shown to be increased in adipose tissue. It has been thought that hyperinsulinemia of these animals contributes to this abnormality. In the present study, we measured the abundance of LPL mRNA in white adipose tissue of three hyperinsulinemic models: Group I: Adult normal rats made hyperinsulinemic via infusion of the hormone (minipumps); Group II: Adult genetically obese fa/fa rats; Group III: Ventromedial hypothalamic (VMH)-lesioned rats. In all these models of hyperinsulinemia the relative abundance of LPL mRNA measured in adipose tissue was markedly increased compared to their respective normoinsulinemic controls. This was correlated with actual increases in total LPL enzyme activity. Since chronic hyperinsulinemia imposed to normal rats produces an upregulation of adipose tissue LPL mRNA, and as hyperinsulinemia is the most salient endocrine abnormality of Group II to III, it is concluded that hyperinsulinemia plays a significant role in the increased LPL mRNA content and LPL activity observed in the animal models just mentionned.

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EXPRESSION OF THE INSULIN RESPONSIVE GLUCOSE TRANSPORTER (GLUT4) mRNA AND GLUCOSE TRANSPORT IN ZUCKER RATS : A LONGITUDINAL STUDY

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Aim : To study the evolution of expression of GLUT4 mRNA and glucose transport in tissues from 21-day lean and pre-obese, 31-day and 10 weeks old lean and obese Zucker rats. Northern blots werre performed on white adipose tissue (WAT), extensor digitorum longus (EDL), and diaphragm (DIAPH). Hybridization with ^{32}P GLUT4 cDNA enabled autoradiograms to be quantified. Glucose uptake in response to insulin was studied in isolated diaphragm using labelled 2-deoxy-D-glucose (2DG). The abundance of GLUT4 mRNA was 5, 2 and 2 fold greater in WAT of 21-day pre-obese, 31 and 70 day old obese rats respectively, compared to controls. GLUT4 mRNA abundance in 21-day obese DIAPH was 5 times greater than in lean controls and was significantly increased in DIAPH from 31-day obese rats, with no intergroup difference at 10 weeks. Similar results were obtained for EDL. Basal glucose uptake in diaphragm was significantly greater in 21-day obese pups compared to lean whilst there was no difference in the other two groups. The 2DG dose-response curve to insulin was upward shifted for diaphragm of 21-day pups compared to lean. That of 31-day and 10 weeks rats were downward shifted. Hence, insulin resistance starts only after weaning, being a progressive process that evolves more rapidly in muscles than in adipose tissue.

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HYPERINSULINEMIA INCREASES THE AMOUNT OF GLUT4 mRNA IN WHITE ADIPOSE TISSUE (WAT) AND DECREASES THAT OF MUSCLES

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Aim : To investigate the impact of hyperinsulinemia imposed on normal rats on the in vivo glucose utilization by white adipose tissue and muscles, and on the amount of GLUT4 mRNA and protein in these tissues. Lean rats were implanted with osmotic minipumps delivering 2 IU of insulin per day for 4 days, or saline. In one group glucose was infused to prevent hypoglycemia. At the end of respective treatments, the in vivo glucose utilization of individual tissues was measured during euglycemic-hyperinsulinemic clamps associated with the 2-deoxy-D-[1-^{3}H] glucose technique. Parametrial WAT, the diaphragm and the tibialis were removed for measurements of the abundance of GLUT4 mRNA and GLUT4 protein. The insulin-stimulated glucose utilization of insulin treated, or of insulin plus glucose treated rats, was increased in white adipose tissue and decreased in several muscles compared to controls. In insulin-treated rats the abundance of GLUT4 mRNA was increased in WAT and decreased in muscles; the amount of GLUT4 protein (monoclonal antibody) was increased in white adipose tissue and decreased in tibialis. Conclusion: These data favor the view that hyperinsulinemia is a driving force responsible for increased white adipose tissue metabolic activity together with muscle insulin resistance.

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TRANSIENT, STIMULATORY EFFECT OF CRF ON INSULINEMIA AND IMPACT ON ORAL GLUCOSE TOLERANCE IN LEAN AND GENETICALLY OBESE fa/fa RATS

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The effects of intracerebroventricular (i.c.v.) CRF administration on plasma glucose and insulin levels were investigated in lean Zucker (FA/?) and genetically obese fa/fa rats. I.c.v. CRF produced a rapid (within one minute), marked but transient increase in insulinemia that occurred without any change in glycemia. The amplitude of the CRF effect on insulinemia was similar in lean and obese rats, since plasma insulin levels doubled at 1 minute in both cases, compared to basal insulinemia. The effect of i.c.v. CRF in stimulating plasma insulin levels was dose-dependent and could be blocked by acute pretreatment of both lean and obese rats with atropine methylnitrate. Similar results were obtained when studying the effects of i.v CRF administration. Furthermore, an i.v. bolus of CRF given to obese animals ameliorated their glucose intolerance and decreased their insulin oversecretion, without affecting these parameters in the lean group. Thus, i.v. CRF administration seems to be able to elicit cephalic-like phase of insulin secretion that is vagally-mediated. Such phase of insulin secretion may be abnormal in genetically obese fa/fa rats, when mimicked by i.v. CRF administration, it is able to normalize their glucose intolerance.

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MECHANISMS OF PHYSIOLOGICAL INSULIN PROCESSING.

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Studies of insulin processing have in general relied on non-authentic tracers and have not been able to describe precisely the fragments formed by degradation of the molecule. We have developed analytical techniques to characterize the fragments generated from tritiated insulins in various biological systems. Thus the labelled insulins have been used to investigate the insulin fragments present in the blood of rats after injection *in vivo*, in perfused rat livers, and after incubation with blood and plasma *in vitro*. The insulin fragments identified in each situation can be compared with those generated by the enzyme insulin proteinase *in vitro*. In the circulation, fragments are found which have been cleaved between residues A12-A13, A13-A14, B10-B11, B11-B12 and B14-B15. In the liver cleavages are observed between residues A13-A14, B10-B11 and B14-B15. The results suggest that insulin proteinase is involved in the generation of fragments observed in the circulation *in vivo* and in the perfused liver. However, the attack on the substrate is either modified from the situation *in vitro* or other enzymes are also involved. In blood and plasma *in vitro*, the fragments are identical to those generated by insulin proteinase *in vitro*.

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CORTICOSTERONE-INDUCE INSULIN RESISTANCE

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We have reported previously that genetically obese and insulin resistant Zucker (fa/fa) rats have an abnormal regulation of the hypothalamo-pituitary-adrenal axis resulting in higher than normal plasma corticosterone levels, both in the resting state and following exposure to stress. To substantiate a role of increased corticosteronemia upon the establishment of insulin resistance, normal Zucker (FA/FA) rats were subcutaneously implanted for 2 days with pellets delivering corticosterone to obtain an increase in the plasma level of the hormone (controls 12±4; corticosterone-treated 387±58 ng/ml), approximatively comparable to the difference observed between lean and obese rats after an immobilization stress (lean 456±19; obese 720±27 ng/ml). Such treatment was associated with an increase in the plasma levels of glucose (controls 116±2; corticosterone-treated 131±7ng/ml) and insulin (controls 1.2±0.1; corticosterone-treated 3.9±0.5ng/ml) indicative of a state of insulin resistance. Control and corticosterone-treated rats were then tested during euglycemic-hyperinsulinemic clamps. It was found that overall glucose handling was halved by corticosterone treatment, due to a marked inability of insulin to shut off hepatic glucose production and to a marked insulin resistance at the level of the glucose utilizing tissues (eg tibialis and EDL).

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TOWARDS THE STUDY OF INSULIN DEGRADATION IN MAN USING MASS SPECTROMETRY

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In order to work in man without radioactivity, we propose to use insulins labelled with stable isotopes (eg. deuterium, nitrogen-15). Such insulin derivatives of different molecular weights are authentic (the native structure is rigorously maintained), non radioactive and can easily be distinguished from endogenous insulin by mass spectrometric analysis.

Several labelled insulins have been prepared and their isolation from plasma samples investigated. Preliminary results concerning preparation, isolation and mass spectrometric analysis will be presented.

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SELECTIVE LABELLING AND INACTIVATION OF CREATINE KINASE ISOENZYMES BY THE THYROID HORMONE ANALOGUE N-BROMOACETYL-L-T_3

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In a previous study, incubation of rat heart mitochondria with ^{125}I-labelled N-bromoacetyl-3,5,3'-triiodo-L-thyronine ($BrAcT_3$), a thyroid hormone analogue with an alkylating side chain, resulted in the selective labelling of a protein doublet around M_r 45'000 on SDS-polyacrylamide gels (Rasmussen et al., FEBS Letters 255, 385-390, 1989). Now, this protein doublet could be identified as mitochondrial creatine kinase. Immunoblotting experiments with the cytoplasmic and mitochondrial fractions of rat heart, brain, and liver, as well as inactivation studies with the purified chicken CK isoenzymes further demonstrated that indeed all four CK isoenzymes (Mi_a-, Mi_b-, M-, and B-CK) were selectively labelled by $BrAcT_3$. In contrast to their bromoalkyl derivatives, thyroid hormones themselves did not compete for CK labelling suggesting that not the thyroid hormone moiety but rather the bromoacetyl-driven alkylation of the highly reactive "essential" thiol group of CK accounts for this selective labelling. Therefore, bromoacetyl-based reagents may allow covalent modification and inactivation of CK isoenzymes in vitro and in vivo.

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CELL CYCLE-SPECIFIC PHOSPHORYLATION OF POLYOMA MIDDLE-T

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Expression of middle-T antigen is essential for transformation by polyomavirus. It associates with cellular tyrosine kinases like $pp60^{c\text{-}src}$. Complex formation leads to increased tyrosine kinase activity. During the cell cycle the activity of $pp60^{c\text{-}src}$ is elevated in mitosis and the protein is phosphorylated in the N-terminal domain. This phosphorylation can be detected as a shift in electrophoretic mobility (see Kaech et al.). We could also observe a shift in the electrophoretic mobility of polyoma middle-T in lysates made from synchronized middle-T-expressing cells. Middle-T showed an increase in the apparent M, in G2 and mitosis and reverted to its normal apparent M, upon entry into G1.

The kinase responsible for these phosphorylations is most likely $p34^{cdc2}$. We found two consensus sequences for phosphorylation by $p34^{cdc2}$ in middle-T. V8 maps of *in vitro* phosphorylated middle-T protein suggest that thr 162 is the site transiently phosphorylated. The shift in electrophoretic mobility of middle-T could be reverted by treating immunoprecipitates of mitotic cells with the ser/thr-specific phosphatase Pase2A (a kind gift by J. Goris & X. Cayla).

We now use site-directed mutagenesis to investigate the role of the putative cdc2 phosphorylation sites in complex formation between middle-T and cellular proteins like Pase2A, $pp60^{c\text{-}src}$ and PI-3-kinase.

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CDC2 KINASE COMPLEXES: CELL CYCLE DEPENDENT CHANGES IN SUBCELLULAR LOCALIZATION OF CYCLINS IN SOMATIC CHICKEN CELLS

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Cyclins are proteins whose abundance fluctuates during the cell cycle and which periodically associate with p34cdc2, a key regulator of the eukaryotic cell cycle. Complex formation between p34cdc2 and cyclin proteins constitutes a major element in the regulation of the cdc2 kinase activity. Most, if not all eukaryotes, contain several types of cyclins, giving rise to cyclin-p34cdc2 complexes which differ in their temporal pattern of activity and, presumably, substrate specificity. To understand the regulation and function of various cyclin p34cdc2 complexes it will be important to determine their subcellular distributions. In order to address this issue, we have cloned and sequenced cDNAs for several different chicken cyclins. Antibodies were then raised against the corresponding proteins expressed in E.coli and utilized to study the biochemical properties and subcellular distribution of cyclin proteins.

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INDUCTION OF PREMATURE MITOSIS BY EXPRESSION OF MUTATED FORMS OF $P34^{cdc2}$ KINASE IN HUMAN CELLS.

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Entry of eukaryotic cells into mitosis is accompanied by dramatic changes in morphology and nuclear organization, and requires activation of $p34^{cdc2}$, an evolutionarily conserved protein kinase. The activity of this kinase is controlled by several mechanisms, including phosphorylation. We have identified several cell cycle regulated phosphorylation sites on chicken $p34^{cdc2}$ and shown that activation of $p34^{cdc2}$ kinase at the G2/M transition occurs concomittant with dephosphorylation of residues Thr 14 and Tyr 15. By site-directed mutagenesis and transfection assays, we have now studied the effects of replacing Thr 14 and Tyr 15 by non-phosphorylatable residues (i.e. alanine and phenylalanine, respectively). When compared to wild type $p34^{cdc2}$ or to mutation of Thr 14 only, mutation of either Tyr 15 alone or Tyr 15 in combination with Thr 14 resulted in increased histone H1 kinase activity of $p34^{cdc2}$ in vitro. Most interestingly, human cells transfected by these latter mutants displayed several features characteristic of mitotic cells, including rounding up, lamina disassambly and premature chromatin condensation.

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ANALYSIS OF THE FUNCTION OF THE FISSION YEAST cdc10 GENE IN THE EXECUTION OF THE START CONTROL

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The cdc10 gene of the fission yeast Schizosaccharomyces pombe is required for the execution of the start control in late G1. In order to better understand the function of this gene in the traverse of the start control, we have isolated extragenic suppressors of a conditional cdc10 mutation, which we are presently characterising. We are also using in vitro mutagenesis techniques to try to isolate dominant lethal alleles of the gene. We performed random mutagenesis with hydroxylamine of a plasmid which expresses the cdc 10 gene under the control of an inducible promoter. Of 15'000 colonies screened to date, one has been isolated which shows a dominant cdc arrest phenotype following induction of the mutated DNA. The nature of the mutation and its effects upon the cell cycle are being characterised. The cdc10 protein also contains two copies of a loose 33 amino repeat found in a number of other proteins which are involved in regulation of transcription in the cell cycle, or in the determination of cell fates during development, or in cellular structure. In order to assess the role of these repeats in the function of the cdc10 protein, we are performing directed in vitro mutagenesis of these regions.

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CHARACTERIZATION OF TWO GENES ENCODING MEMBRANE ASSOCIATED NUCLEAR PROTEINS OF YEAST

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We purified two membrane associated proteins (42kD and 45kD) which cross-react with the monoclonal antibody RL1 generated against rat nuclear pore preparations (Snow et al.,JCB 104,1143). The corresponding genes have been isolated and sequenced. The ORF encoding the 45kD protein contains a hydrophobic N-terminal leader sequence and a transmembrane domain close to the C-terminus of the protein. We postulate that the 45kD protein is a transmembrane protein. Preliminary studies indicate that both proteins are located in or at the periphery of the nucleus. The 45kD protein is essential for mitotic growth. The absence of the 42kD protein leads to reduced growth rate on glucose and to the inability to grow on glycerol.

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INDUCTION OF M-PHASE ENTRY OF PROPHASE-BLOCKED MOUSE OOCYTES THROUGH MICROINJECTION OF OKADAIC ACID, A SPECIFIC PHOSPHATASE INHIBITOR

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We report that a specific inhibitor of types 1 and 2A phosphatases, okadaic acid (OA), induces germinal vesicle break down (GVBD) and chromosome condensation when microinjected into denuded mouse oocytes maintained in prophase block by analogs of cAMP, inhibitors of phosphodiesterase, or a tumor-promoting phorbol ester. GVBD and chromosome condensation are also observed when incompetent oocytes are similarly injected with OA, this effect being dependent on the oocyte diameter. Marked changes in cell shape, cytoskeletal organization, and chromosome condensation with abnormal or abortive spindle formation are associated with such injections. The polar body is not formed. These re sults led to the conclusions that in mouse oocytes, OA acts distal to both the cAMP-modulated pathway involved in meiotic arrest and the inhibitory action exerted by tumor-promoting phorbol esters.

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POTENTIAL ROLE OF THE p53 TUMOR SUPPRESSOR GENE IN THE TUMORIGENESIS OF HUMAN GLIOBLASTOMA

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Cytogenetic and RFLP studies have shown that frequent deletion events occur on chromosome 17p in human astrocytomas and glioblastomas. An interesting candidate gene localized in this region is the tumor suppressor gene p53. This gene product was discovered by its ability to bind tumor promoting viral proteins such as large T antigen of SV40, E1B of Adenovirus type 5 and E6 of human papillomaviruses types 16 and 18. Wild type p53 was shown to act negatively on transformation of rodent fibroblasts by myc and ras oncogenes. We have amplified highly conserved regions of the p53 gene of 5 human glioblastoma cell lines using the PCR. We identified different mutations in exons 5 and 7 by sequencing. In order to determine the mechanism of the p53 gene product action we introduced stably an inducible WT p53 gene into these cell lines. We studied the effect of WT p53 expression in these transformed cell lines in regard to cell growth, transfection efficiency and anchorage-independent growth.

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POST-TRANSCRIPTIONAL REGULATION OF TRANSFERRIN RECEPTOR AND 4F2 ANTIGEN HEAVY CHAIN DURING GROWTH STIMULATION

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Transferrin receptor (TR) and 4F2 antigen (4F2) mRNA are highly expressed in proliferating cells, but virtually absent from quiescent tissues. In addition, TR expression is strongly influenced by the intracellular iron level. Low iron activates cytoplasmic iron regulatory factor (IRF), which stabilizes TR mRNA 10-20 fold upon its binding to hairpins in the 3' untranslated region. It has remained unclear whether this control is sufficient for the proliferation-dependent induction of TR. We, therefore, investigated the mechanisms regulating TR and 4F2 expression during cell growth activation of the IL-2 dependent T cell line B 6.1. When these cells are deprived of IL-2 they arrest in G1 and reduce their TR and 4F2 mRNA expression. The decrease cannot be prevented by activating IRF with the iron chelator desferrioxamine. Moreover, upon iron chelation, IRF activity in arrested B 6.1 cells increases without affecting the TR mRNA levels. These results suggest an additional iron-independent regulatory mechanism at the onset of cell proliferation. However, as determined by nuclear run-on assays transcription rates in the TR and 4F2 gene were not significantly different in arrested and restimulated cells. We conclude that during the onset of cell proliferation TR and 4F2 expression are regulated by a post-transcriptional mechanism.

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CALRETININ EXPRESSION IN WIDr CELLS

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Since cell biological and biochemical studies would be facilitated by the availability of cells expressing calretinin "in vitro", we screened cell lines for the presence of this calcium binding protein. Strong calretinin immunoreactivity was observed in cells undergoing mitosis and deriving from three different tumoral-lines (WiDr, OVGE-1 and PC12). The distribution of immunoreactivity varied according to the mitotic phase: in prophase and telophase it was homogeneously diffused in the cytoplasm, whereas in metaphase and anaphase it was exclusively localized at the level of spindle microtubules. Chromosomes were always negative. Some interphase cells were also immunoreactive, their percentage being higher in the WiDr cell-line. These morphological observations have been strenghtened for WiDr-cells by immunoprecipitating extracts with specific antibodies against calretinin and by cloning the calretinin cDNA. The presence of calretinin in the mitotic spindle could be related to the molecular mechanism controlling the assembling/disassembling of microtubules during spindle elongation and chromatide separation.

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INCREASED FIBRONECTIN mRNA LEVELS IN SV40 AND POLYOMA INFECTED MOUSE CELLS.

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Simian virus 40 and polyomavirus induce a mitotic host reaction in G_0-arrested mouse kidney cells. To study alterations in cellular gene expression during virus-induced S-phase, we did a differential screening of a mouse kidney cDNA library with probes prepared from mRNAs of virus-infected and mock-infected cells. We characterized cDNA recombinant pKT13 which detected increased mRNA levels in cells infected by SV40 or polyoma virus. Sequence analysis revealed 100% homology with the 3'-end of the mouse fibronectin (FN) gene. High levels of FN mRNA were found in confluent, i.e. G_0-arrested mouse kidney cells even after prolonged culturing, while no FN mRNA was detected in the proliferating cells and decreased levels in SV40-transformed mouse kidney cells. Since FN mRNA increased after virus infection, as did many other cellular mRNAs, the decrease or repression of FN gene expression in proliferating cells was apparently not directly linked to the proliferative state of the cells.

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INTERIM STORAGE OF A NUCLEAR MATRIX PROTEIN ALONG VIMENTIN DURING MITOSIS

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A monoclonal antibody (M2) directed against the high molecular weight neurofilament component (NF-H) crossreacts with a nuclear matrix (NM) protein of 185kD (P185). Immunofluorescence studies of mitotic cells in culture (M617) revealed a dynamic cell-cycle dependent behavior: [1] disassembly in prophase, [2] cytoplasmic release, [3] cytoplasmic reassembly and [4] transport to the telophase nuclei. Combining in situ fractionation of the cells in culture including detergents, DNase I, 2M NaCl and RNase A treatment with SDS-PAGE analysis of the eluted proteins, we have demonstrated that P185 belongs to the NM. Immunodot-tests of the cytoskeletal fraction under assembly and disassembly conditions for vimentin in vitro revealed a cosedimentation of vimentin with P185. In double IF stainings with an anti-vimentin mab and mab M2 we could demonstrate that most of the vimentin filaments remain intact during mitosis and that P185 is colocalized with the perinuclear vimentin basket in prophase and metaphase cells. From our biochemical and morphological data we concluded that at least during the first mitotic stages the nuclear matrix protein (p185) is stored along vimentin filaments.

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HUMAN NTS-1 TUMOR SUPPRESSOR : NUCLEOTIDE SEQUENCE AND FUNCTIONAL ANALYSIS BY SITE-DIRECTED MUTAGENESIS

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Previously we have molecularly cloned the candidate human NTS-1 suppressor on an 18 kb DNA restriction fragment. Transfer of this sequence into H-ras transformed rat FE-8 cells results in suppression of anchorage independence and a reduction of tumorigenicity. A genomic subclone harboring 2.5 kb of human NTS-1 DNA is sufficient to induce reversion of FE-8 cells. We have determined the nucleotide sequence of human NTS-1 DNA. Based on the genomic sequence, primers were synthesized and used to isolate NTS-1-specific cDNAs from mRNA of FE-8 revertant cells by reverse transcription and PCR amplification. Three open reading frames encoding possible peptides of 13 to 56 amino acids were identified. The start codons of each of the predicted peptides were modified by site-directed mutagenesis (ATG TTG mutation). The functional activity of each DNA construct was assayed by transfection into FE-8 cells. All mutated DNA sequences were able to confer anchorage-dependence on H-ras transformed FE-8 cells. We conclude that none of the predicted peptides is the product of the NTS-1 suppressor. Suppression of the neoplastic phenotype is probably caused by a different mechanism.

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ASSOCIATION OF PP60$^{c\text{-}SRC}$ WITH POLYOMAVIRUS MIDDLE-T ABROGATES MITOSIS-SPECIFIC ACTIVATION

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The *in vitro* activity of the cellular tyrosine kinase pp60$^{c\text{-}src}$ is increased up to 10fold in mitotic compared with interphase cells. Activation is accompanied by N-terminal thr and ser phosphorylations which also account for the observed shift in the electrophoretic mobility of this protein. In normal cells, kinase activity reverts to a normal level within minutes after release from a mitotic block into G1.

Polyomavirus middle-T forms a stable complex with and activates pp60$^{c\text{-}src}$. Middle-T-associated pp60$^{c\text{-}src}$ is not phosphorylated at tyr 527, a site negatively regulating src kinase activity. Here we show that in mitotic polyomavirus-transformed cells, pp60$^{c\text{-}src}$ is not further activated. Furthermore, we investigated a cell line transfected with a transforming c-src mutant carrying phe instead of tyr in position 527. This mutant protein shows the indicative electrophoretic shift in mitosis, suggesting that it is still transiently phosphorylated in the aminoterminus, yet its activity is constitutively high throughout the cell cycle. N-terminal phosphorylation of pp60$^{c\text{-}src}$ is therefore required but not sufficient for mitotic activation. Tyr 527 is transiently dephosphorylated in mitosis. We suggest that cell transformation by middle-T or pp60$^{c\text{-}src(527F)}$ is the result of constitutive activation of pp60$^{c\text{-}src}$ in the cell cycle and leads to aberrant phosphorylation of critical substrate proteins at tyrosine residues.

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SV40/POLYOMA HYBRID VIRUSES EXPRESSING INDIVIDUAL POLYOMA T ANTIGENS.

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To define the role and the mode of action of individual polyoma T antigens for induction of S-phase and mitosis in quiescent cells, we constructed a series of SV40/polyoma chimeric viruses expressing individual polyoma T antigens. These viruses are propagated on *COS cells expressing* SV40 early proteins. Homogeneous virus preparations with titers between 10^6 and 10^7 infectious particles per ml were obtained. Immunofluorescence and immunoprecipitations showed that these viruses express the expected polyoma proteins in COS cells. Currently we modify the constructs to improve gene expression in other cells. Such altered SV40 viruses would be an ideal means to introduce efficiently and to test the biological activity of potential nuclear mitogens in quiescent cells.

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FUNCTIONAL ANALYSIS OF CALCYCLIN BY CELL TRANSFECTION EXPERIMENTS

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Calcyclin is an EF-hand calcium-binding protein of the highly conserved S-100 protein family. It has been proposed that members of this family might function intracellularly to induce cell differentiation and proliferation, possibly by modulation of membrane-cytoskeleton interaction or by microtubule polymerization and depolymerization, and even extracellularly to promote neurite extension. However, it is not known if a common mechanism underlies these postulated divergent functions of the S-100 protein family.

We isolated calcyclin from a human epithelial carcinoma cell line and confirmed its partial amino acid sequence by Edman degradation and mass spectrometry. The protein is expressed in different human cell lines deriving from epithelial cells, neuroblastomas and myoblasts, as shown by Western blot analysis. Calcyclin mRNA has been identified in different cell lines as a product accumulating during G1 phase. Thus a role in cell cycle regulation has been postulated. To test this hypothesis we amplified calcyclin cDNA by PCR from a human brain lambda library, cloned it into the expression vector pRc/CMV either in sense or antisense orientation, and transfected the constructs into a human neuroblastoma cell line. Different subclones were isolated and are being characterized with respect to calcyclin expression and morphology. Their proliferation rate is examined by flow cytometry. Preliminary results show a marked morphological difference between the subclones transfected with sense and those transfected with antisense cDNA.

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Topoisomerase II is Phosphorylated and Regulated by Casein Kinase II

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DNA topoisomerase II is an essential nuclear enzyme which controls DNA topology by introducing transient double strand breaks that relax both positively and negatively supercoiled DNA. Studies with topoisomerase II mutants in the yeast *S. pombe* have established that this enzyme is required to condense and separate intertwined sister chromatids at early and late mitosis. *In vitro* the decatenating activity of topoisomerase II can be modulated by phosphorylation by a number of kinases, including, protein kinase C, casein kinase II, and the cdc2-p34 kinase. In yeast and other organisms the level of topoisomerase II phosphorylation *in vivo* varies significantly through the cell cycle, peaking in mitosis. The major kinase that phosphorylates topoisomerase II both in G1 and in M phase *in vivo* is casein kinase II, as demonstrated by phosphopeptide patterns and phosphorylation analysis in a casein kinase II ts mutant. The same serine and threonine sites are phosphorylated *in vivo* as *in vitro* by casein kinase II and most of these are in found in the less conserved C-terminal tail of the protein. This phosphorylation does not alter the sequence specificity for topoisomerase II-mediated double stranded cleavage. The means by which this phosphorylation modulates the enzymatic activity is under study.