c-*ets*-2 and the Mitogenic Signal Pathway

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A. Introduction

The feature that defines a cancer cell is its ability to propagate under conditions that typically inhibit the growth of the normal cell. In cells where the genetic constitution remains unaltered, restraints on growth are imposed by regulatory activities that take place at the cell surface. These activities, which may be due to either cell contact or a decrease in the production and availability of growth factors, are communicated through the cytoplasm to the nucleus, thus regulating the synthesis of messenger RNAs essential for the unique proteins needed to initiate subsequent DNA synthesis and mitosis. Cells can be relieved of this barrier to mitosis by cell dispersal or addition of growth factors; this molecular environmental change is similarly recognized at the surface of the cell, and is transmitted through the cytoplasm to the nucleus. This signal transduction process activates the transcription and synthesis of specific proteins and other macromolecules that trigger the mitotic process. The process and events leading to cell division are called here the "mitogenic signal pathway".

I. Oncogenes

The RNA tumor viruses (retroviruses) have been particularly useful in identifying and dissecting the metabolic pathways associated with mitosis, as well as for the recognition of macromolecules that are potential determinants of the malignant process [1]. These viruses were found in naturally occurring tumors (although extremely rare) in mice and chickens, it was proposed that such viruses could be components of all cells, and that the part of the virus encoding transforming activity, known as the oncogene, was responsible for the genesis of tumor cells. Since that time, the term "oncogene" has evolved and has been expanded to include all transforming genes in retroviruses that have nucleotide sequences homologous to cellular sequences, as well as all cellular genes that have transforming capability in DNA transfection assays. The complete cellular gene from which the transforming oncogene is derived is called the "proto-oncogene".

Molecular clones of oncogenes have provided an excellent opportunity to examine and compare the structural features of cellular proto-oncogenes in great detail. The general conclusion derived from these studies is that most viral oncogenes are truncated, mutated, or otherwise modified versions of normal cellular proto-oncogenes. The most exciting result of these comparative analyses is the recognition that several oncogene proteins can be identified as altered variants of normal cellular proteins that are involved in signal transduction and growth regulation processes.

II. Oncogenes Are Components of the Mitogenic Signal Pathway

The identification of oncogene to proto-oncogene product and the recognition of their relevance to components of the sig-

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nal transduction pathways have allowed researchers to create a cellular paradigm for the normal mitogenic pathway and helped them to understand how disruption or amplification of the normal pathway can result in tumorigenesis.

The extensive molecular cloning and analysis of the retroviral oncogenes has allowed their grouping according to their biological function, as shown in Fig. 1. Here we see that the oncogenes can be characterized as growth factors (platelet-derived growth factor and *v-sis*), growth factor receptors (epidermal growth factor receptor and *v-erbB* or colony-stimulating factor-1 and *v-fms*), tyrosine kinases (*src* family of kinases), G-proteins (*ras*), and transcriptional activators (*fos, jun/AP-1*). The ligand-responsive transcription factors form a special class, in that these receptors bind their ligand directly and are then able to activate the corresponding genes (thyroid hormone receptor *erbA* and *v-erbA*).

III. Growth Factor Receptor

The prototype receptor, for which the epidermal growth factor (EGF)-receptor is one example, binds growth factor in its external domain. This region is typically located in the amino-terminal half of the protein molecule, a region that is highly glycosylated. Several receptors are known to have an abundance of cysteine residues that are believed to stabilize the conformational structure of the molecule. Although receptors may be specific for individual growth factors, it is likely that certain receptors will respond to more than one ligand. For example, the receptor for EGF can bind with equal affinity to (at least) three different peptides: EGF, transforming growth factor α (TGFα), and vaccinia virus growth factor [2–4]. The ligands appear to have little amino acid sequence homology in common, and presumably have substantial conformational similarities, since they can bind equivalently to the same receptor.

The transmembrane domain of receptors contains a significant proportion of hydrophobic amino acids; such sequences are characteristic of a membrane-associative capability. In addition to being a determinant in membrane localization, the structure of this region may also be important to its receptor activation function; a single amino acid replacement in this hydrophobic region converts a normal receptor molecule into a transforming protein.

The cytoplasmic domain of the receptor appears to have the potential for enzymatic activity; in several cases at least

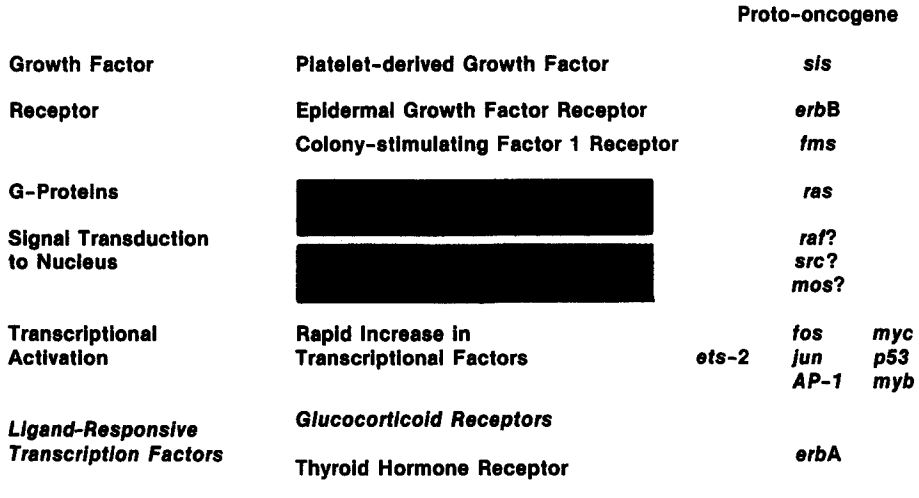

Fig. 1. Signal transduction cascade: The molecular cloning and sequencing of the viral oncogenes has allowed the definition of ■ their corresponding proto-oncogenes. These proto-oncogenes fall into the categories shown. The *black boxes* represent unknown mechanisms but are involved in the mitogenic signal pathway. The ligand-responsive transcription factors are a special class of proteins which are able to bind to sequence-specific elements after ligand binding

this activity is a tyrosine kinase. This internal polypeptide portion of the receptor is phosphorylated at specific serine and threonine residues by protein kinase C, as well as on its tyrosine residue by autophosphorylation. These phosphorylations may, in turn, affect and regulate the kinase activity of the receptor. It appears that ligand binding can activate the kinase activity of receptors. It is known that truncated receptor molecules, as represented by certain oncogene-encoded proteins, are devoid of the binding domain; these defective receptors are constitutively, enzymatically active and independent of growth factors [5].

IV. G-Proteins and *ras*

The activated tyrosine kinases can transmit their effect to nuclear events by either a *ras*-sensitive or a *ras*-insensitive pathway. The *ras*-insensitive pathway is not very well understood but may involve other members of the *scr* family of protein kinases. The *ras*-sensitive pathway involves one of the three *ras* genes (H-*ras*, K-*ras*, and N-*ras*) identified. The products from these genes localize to the cytoplasmic side of the plasma membrane, similar to the subcellular location of G-proteins [6]. Additionally, *ras* and G-proteins have highly conserved amino acid residues at their GTP-binding sites. However, no other amino acid homologies exist between these two classes of proteins. Therefore, while some functional similarities appear to exist for *ras* and the other G-proteins, each class of protein remains clearly distinct. Stimulation of a receptor results in the activation of the G-protein by releasing bound GDP and then binding of cellular GTP. The *ras*-sensitive pathway implicates phospholipid metabolism with generation of second-messenger molecules such as inositol triphosphate (IP3) and diacylglycerol (DG). The IP3 leads to release of calcium from the endoplasmic reticulum. The DG, along with free calcium, will activate protein kinase C [7]. The protein kinase C then could transmit its effect to the nucleus by poorly characterized processes shown by the black boxes in Fig. 1. In fact, the *ras*-sensitive and *ras*-insensitive pathways may converge and use a similar mechanism to activate nuclear regulatory proteins.

Since *ras* proteins bind guanosine nucleotides, it has been strongly suggested that they are related to G-proteins. In the active conformation, the G-protein is able to regulate second messages (cyclic adenosine monophosphate-cAMP, cyclic guanosine monophosphate-cGMP, DG, IP3, and Ca^{++}) by activating or inhibiting the enzymes responsible for their production. Hydrolysis of the bound GTP to GDP returns the activated G-protein to an inactive state, eliminating its regulatory effect. The recently discovered guanosine triphosphatase protein (GAP) regulates the activated *ras* by promoting GTPase activity [8] and may represent a cellular effector molecule for *ras*.

The *ras* oncogene stimulates the mitogenic signal transduction pathway. Rodent cells transformed by v-*ras* exhibit increased levels of both phosphatidylinositol diphosphate (PIP2) and its second messages DG and IP3 [9]. Consistent with this finding is the observation that following microinjection of a transforming $p21^{ras}$ protein into frog oocytes, rapid increases of PIP2, IP, IP2, and particularly DG were seen [10]. Microinjection of a monoclonal antibody to 21^{ras} that effectively neutralizes intercellular $p21^{ras}$ activity prevents the mitogenic activity of a phorbol ester or a calcium ionophore [11], suggesting that *ras* oncogene-encoded proteins can function at more that one critical site in the signal transductions pathway.

V. Nuclear Proto-oncogenes

The nuclear proto-oncogenes share several characteristics, including low abundance, rapid turnover, post-translational modification such as phosphorylation, response to mitogenic stimuli, and DNA binding. The best characterized of these proteins is *fos*, which has recently been

shown to be co-induced with the transcription factor *jun/AP-1*. The *jun* binds to a specific DNA sequence (tgactca) found in phorbol ester (TPA)-responsive genes, and the *fos* binds to the *jun*, probably by protein-protein interactions, to begin forming an active transcriptional unit [12].

VI. The Mitogenic Response in Wound Healing

An example of how the mitogenic response pathway works in vivo is given for the platelet-derived growth factor (PDGF), which is found in alpha granules of the circulating platelets and probably functions in the repair of blood vessels in wound healing [13]. Thus, on wounding, platelets adhere to the blood vessel walls and PDGF is released, stimulating the migration of smooth muscle cells from the medial and intimal layers of the artery, where these cells proliferate in response to the injury and subsequent release of PDGF. The presence of the growth factor causes the proliferation of the cells necessary for wound healing, and as the repairs are made, the source of PDGF is removed (the clot disappears) and cell proliferation stops. Thus, the growth factor binds to the PDGF receptor and activates its associated tyrosine kinase activity, resulting in G-protein activation of phospholipase C, which hydrolyses phosphatidylinositol, generating the second messengers IP3 and DG. The IP3 is responsible for the release of Ca^{2+} from the endoplasmic reticulum, and the calcium along with DG can activate the protein C kinase [9]. The DG can be further metabolized by kinases to phosphatidic acid, or by phospholipase A_2 to liberate arachidonic acid – a precursor of prostaglandins. The activation of protein kinase C rapidly induces a nuclear response through transcriptional activation of *fos* and *myc* [14, 15]. The proto-oncogene *ras* is involved in this part of the pathway, since microinjection of monoclonal antibodies to the *ras* protein can block the mitogenic response elicited by phosphatidic acid or by prostaglandin f1α [11], indicating that *ras* may function by coupling a product(s) of phospholipid with cytoplasmic factors, which in turn can activate nuclear regulatory proteins.

B. *ets*-2 and the Mitogenic Signal Pathway

Our interest in the mitogenic signal transduction pathway comes from our investigations on the human *ets*-2 gene [16–22]. This gene has a high degree of homology to the viral oncogene v-*ets*, which was originally identified as a cell-derived sequence transduced into its genome by the avian leukemia virus, E26. Previous observations made in our laboratory have suggested that the *ets*-2 gene has a role in cell proliferation [23, 24]. The product of the *ets*-2 gene is preferentially expressed in a wide variety of proliferative tissue and the level of *ets*-2 expression was generally greater in tissue obtained from a variety of young organs, compared with adult organs of the same type.

We have also studied *ets* expression in quiescent BALB/c 3T3 fibroblasts following serum stimulation; both *ets*-1 and *ets*-2 RNA are increased 3 h after serum addition, while DNA synthesis peaked at 16 h (Fig. 2). The activation of 3T3 cells with serum also increases *fos*, *myc*, mouse metallothionin (MMT) and heat shock protein (HSP) mRNAs. These changes represent transcriptional activation of these genes. In Fig. 3 we show a post-translational mechanism for the activation of the *ets*-2 involving increase in the half-life ($t½$) of the *ets*-2 protein. These two mechanisms are not mutually exclusive, and the preference for the slower transcriptional or faster post-transcriptional mechanisms for regulation of the *ets*-2 may reflect differences in cell type or tissues examined. Transcriptional activation of the nuclear oncogenes has been described for many systems; the post-translational control of protein concentration by prolongation of $t½$ via

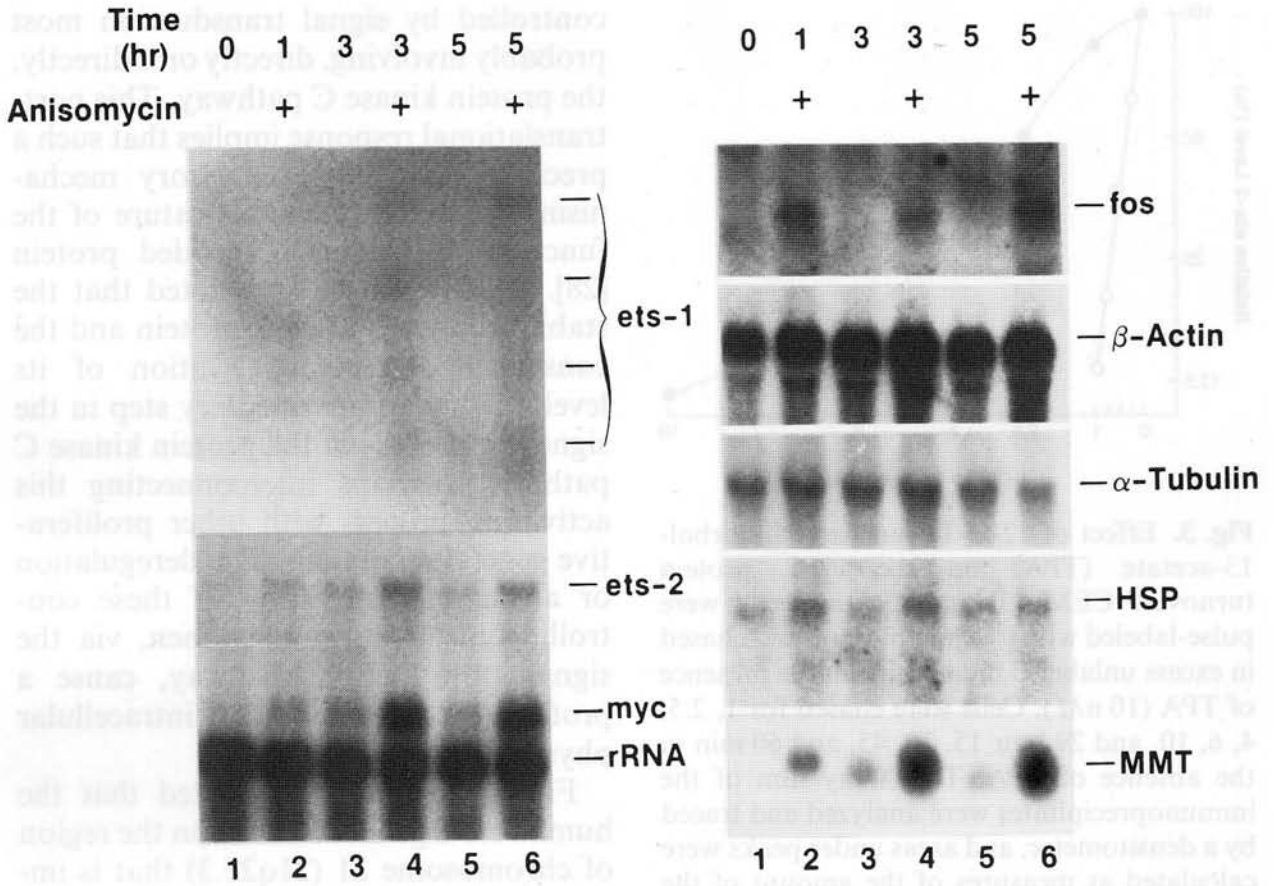

Fig. 2. Gene expression in Balb/c 3T3 fibroblasts after serum stimulation. The *ets*-1 and *ets*-2 mRNA are induced by serum and reach a maximum by 3 h in the presence of the protein synthesis inhibitor, anisomycin. *fos* and heat shock protein (HSP) have reached their maximum at the first time point measured (1 h), while *myc* and mouse metallothionin (MMT) have the same kinetics as do the *ets* genes. The α-tubulin and beta actin housekeeping genes are not induced by this treatment

the protein kinase C pathway is thus far unique for the *ets*-2.

The human *ets*-2 gene product has been identified by means of specific antibodies directed against antigen obtained from the bacterially expressed partial cDNA clone of the *ets*-2 gene [25, 26], as well as an oligopeptide antigen corresponding to a highly conserved hydrophilic region of *ets*. Using both types of sera, a 56-K protein has been identified as the human *ets*-2 gene product; this protein, like the oncogene product $p135^{gag\text{-}myb\text{-}ets}$, is also located in the nucleus. This nuclear localization of the human *ets*-2 protein supports its relationship with other nuclear proto-oncogene products, such as those encoded by c-*fos* and c-*myc*, that have been seen to be expressed in association with cellular proliferation.

Recently, we have found that the *ets*-2 protein is phosphorylated and has a rapid turnover of normally less than half an hour [27, 28]; however, when cells are treated with a tumor promoter such as the phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA), the level of *ets*-2 protein very quickly becomes markedly elevated. This increase in *ets*-2 protein appears to be due to the stabilization of the protein, because the *ets*-2, $p56^{ets\text{-}2}$ product increased its half-life by more than 2 h in the presence of TPA (Fig. 3), while the *ets*-2-specific mRNA did not change. Since an inhibitor of protein kinase C also interferes with the stabilization of $p56^{ets\text{-}2}$, and the effect of TPA could be mimicked by a synthetic diacylglycerol, it appears that the protein kinase C signal pathway is probably involved in the induction of this nuclear

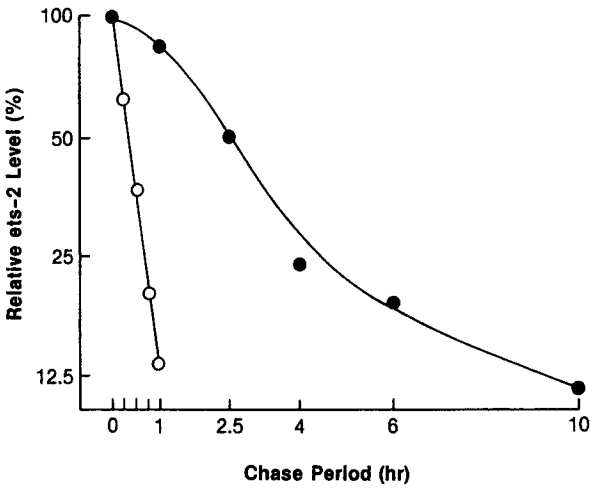

Fig. 3. Effect of 12-O-Tetradecanoylphorbol-13-acetate (TPA) on the *ets*-2 protein turnover. CEM (T-lymphocytic) cells were pulse-labeled with [^{35}S]methionine and chased in excess unlabeled methionine in the presence of TPA (10 n*M*). Cells were chased for 1, 2.5, 4, 6, 10, and 29 h or 15, 30, 45, and 60 min in the absence of TPA. The X-ray film of the immunoprecipitates were analyzed and traced by a densitometer, and areas under peaks were calculated as measures of the amount of the labeled *ets*-2 protein. The labeled *ets*-2 protein levels at various chase periods were normalized to the 100% value obtained at zero time and plotted in semilogarithmic scale. The data were fit to a first-order exponential decay curve with a correlation coefficient of 0.99. *Open circles* are control cells ($t½ = 20$ min); *closed circles* are the CEM cells chased in the presence of 10 n*M* TPA ($t½ = 160$ min)

proto-oncogene. In this respect, however, the *ets*-2 protein is unique from the other nuclear oncogene products in its ability to respond to TPA post-translationally. Other nuclear proto-oncogenes respond to TPA at the mRNA level, and subsequently at the protein level, but thus far, only the *ets*-2 protein level is distinct since it increases in the absence of any increase mRNA level. Additionally, consistent with a post-translational mechanism, it should be noted that the protein synthesis inhibitor cycloheximide enhances the effect of TPA on the level of *ets*-2 protein retarding its turnover even further. Taken together, these data suggest that the expression of the proto-*ets*-2 gene and its encoded products are rapidly controlled by signal transduction most probably involving, directly or indirectly, the protein kinase C pathway. This post-translational response implies that such a precisely controlled regulatory mechanism may be an essential feature of the function of the *ets*-2 encoded protein [28]. It can even be speculated that the stabilization of the *ets*-2 protein and the consequent transient elevation of its level may be an intermediary step in the signaling process of the protein kinase C pathway, perhaps interconnecting this activation process with other proliferative gene(s) regulation. The deregulation or any subtle alteration of these controlling mechanisms may then, via the signal transduction pathway, cause a profound change in the intracellular physiology.

Finally, it should be noted that the human *ets*-2 gene is located on the region of chromosome 21 (21q22.3) that is implicated in Down's syndrome [29–31]; trisomy of this small chromosomal domain results in the full manifestation of the Down's syndrome phenotype. Because the *ets*-2 protein level appears to be under precise control, the increase in the *ets*-2 gene dosage resulting from the trisomy may seriously affect the control of the *ets*-2 protein, and it is therefore conceivable that this deregulation is a contributing factor in the development of Down's syndrome.

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