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PROTEIN KINASE C AND NOVEL RECEPTORS FOR THE PHORBOL ESTERS AND THE SECOND MESSENGER DIACYLGLYCEROL

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Protein kinase C isozymes: a family of receptors for the phorbol ester tumor promoters

The phorbol esters and related diterpenes are natural products isolated from plants of the families *Euphorbiaceae* and *Thymeleaceae*, and have for many years been the preferred pharmacological tools for studying protein kinase C (PKC), a family of serine-threonine kinases that play a central role in the mechanisms of carcinogenesis. The phorbol esters have attracted great interest since many decades ago because of their high potency as tumor promoters in the mouse skin. Early experiments have determined that these compounds mimic the action of diacylglycerol (DAG), a lipid second messenger that can be generated upon activation of seven-transmembrane or tyrosine kinase receptors by extracellular signals. The phorbol esters exert a variety of effects in cells, which include changes in proliferation, malignant transformation, differentiation and cell death.

Our laboratory focuses on the study of the receptors for the phorbol esters and DAG. It was in the early '80s that several groups identified PKC as the receptor for these tumor promoting agents. PKC comprises a family of at least 10 related isoforms classified into three groups, which are subject to different biochemical regulation, intracellular localization and tissue distribution (Fig. 1). The first group, named "classical" or "conventional" PKCs (cPKCs), includes PKCα, βI, βII, and γ. These PKC isoforms can be activated by calcium. The second group, named "novel" PKCs (nPKCs), includes PKCδ, ε, η, and θ. The nPKCs are calcium-independent. The third group or atypical PKCs (aPKCs) are also calcium independent and includes the isozymes PKCζ and ι (the mouse homolog of PKCι has been named PKCλ). A major difference in the regulation of the PKC isozymes is that only the cPKCs and nPKCs are sensitive to phorbol ester/DAG activation. A related kinase, named PKCμ or PKD has been also cloned. PKCμ/PKD is insensitive to calcium regulation, although it binds phorbol esters with high affinity. Because of its unique substrate specificity that completely differs from that of cPKCs, nPKCs and aPKCs, PKCμ/PKD should be considered a distant relative of PKCs. However, PKCμ/PKD is activated in cells by PKC phosphorylation and can therefore function downstream of PKCs.

Multiple roles for PKC isozymes in cell growth and cell death

The complexity of phorbol ester actions is probably related to the presence of multiple cellular phorbol ester/DAG receptors, which include not only PKC isozymes but also other classes of receptors (see below). In most cases, at least five or more PKC isozymes are present in a single cell, which may have overlapping or opposite functions. The overlap in function may result from a relatively poor selectivity of individual isozymes towards cellular substrates. Opposite roles for PKC isozymes in cell growth have been described in fibroblasts, where PKCδ is growth inhibitory and PKCε is growth stimulatory and may even function as an oncogene.

While it was initially established that phorbol esters are mitogenic through PKC activation, these compounds may also inhibit cell growth or induce apoptosis in several cell types. PKC isozymes operate as regulators of the cell cycle both during G1/S progression and G2/M transition. The PKC-mediated signaling pathways that regulate cell growth and cell death are under active investigation and appear to be cell-type dependent.

In our laboratory we are currently investigating the role of PKC isozymes in prostate cancer cells. Interestingly, phorbol esters induce apoptosis in androgen-

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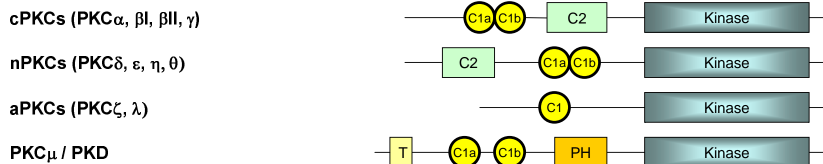
PROTEIN KINASE C ISOZYMES



NOVEL NON-KINASE PHORBOL ESTER RECEPTORS

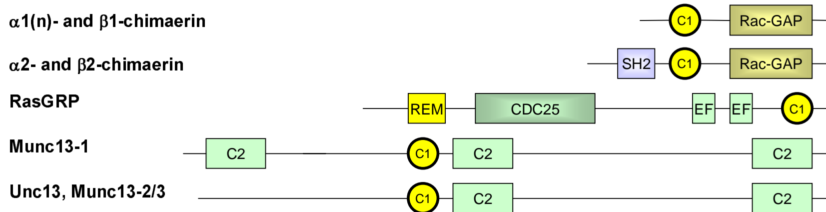


Fig. 1.– Structure of PKC isozymes and novel "non-kinase" phorbol ester receptors. The phorbol esters and related analogs bind to the C1 domain in PKC isozymes (with the exception of aPKCs) and novel "non-kinase" receptors. C1, cysteine-rich domain; C2, phospholipid binding domain (also a calcium binding domain in cPKCs); T, transmembrane domain, PH, PH domain; SH2, SH2 domain; Rac-GAP, Rac GTPase-activating protein domain; REM, Ras exchange motif; CDC25, region with homology to guanine exchange factor domain of Cdc25 and Sos; EF, EF hand.

dependent LNCaP prostate cancer cells but not in androgen-independent prostate cancer cell lines, where inhibition of cell growth is observed upon phorbol ester treatment. To evaluate the involvement of PKCs in phorbol ester-induced apoptosis, we used replication-deficient adenoviruses for PKC isozymes. We found that overexpression of the novel isoform PKCδ using the adenoviral-delivery system induces apoptosis in LNCaP cells. This effect can be blocked by PKC inhibitors, pan-caspase inhibitors, or by overexpression of the anti-apoptotic molecule Bcl-2. Interestingly, the apoptotic effect of PKCδ does not involve the activation of caspase-3, which has been reported to cleave PKCδ with the subsequent release of a constitutively active catalytic fragment. Our studies suggest that in LNCaP cells, phorbol esters activate PKCδ by an allosteric mechanism rather than by proteolytic cleavage. The involvement of PKCδ in phorbol ester-mediated apoptosis is supported by experiments where expression of a dominant-negative (kinase inactive) PKCδ in LNCaP cells blocks phorbol ester-induced apoptosis. Preliminary mechanistic studies in our laboratory suggest that PKCδ-mediated apoptosis in LNCaP cells involves the activation of the p38MAPK pathway, and a deregulation of the Akt/PKB survival pathway.

The inhibitory role of PKCδ in prostate cancer cells is further supported by experiments in soft agar and in nude mice models. In fact, infection of prostate cancer cells with a PKCδ adenovirus reduces the formation of colonies in soft agar (an assay that evaluates anchorage-independent cell growth) and inhibits tumor formation when cells are inoculated into nude mice. These results support the concept that PKC isozymes are attractive targets for pharmacological intervention in cancer chemotherapy.

The binding site for the phorbol ester tumor promoters: the C1 domain

All PKCs have in common a C1 domain (also known as cysteine-rich or zinc finger domain) of 50-51 amino acids located at the N-terminal regulatory region (Fig. 1). Each of these domains possesses the motif $HX_{12}CX_2CX_{13/14}CX_2CX_4HX_2CX_7C$, where H is histidine, C is cysteine, and X is any other amino acid. The C1 domain is duplicated in tandem in cPKCs and nPKCs (C1a and C1b domains). A single copy is present in the phorbol ester unresponsive aPKCs. Through a series of deletional analysis and site-directed mutagenesis, it was established that the C1 domain is the phorbol ester binding site in cPKCs and nPKCs.

PKCs are not the only proteins possessing C1 domains. However, not all of the proteins having C1 domains are phorbol ester receptors. Among the C1 domains unresponsive to phorbol esters are those of the proto-oncogenes c-Raf, Vav and diacylglycerol kinases.

An important finding was the discovery of novel receptors for the phorbol ester tumor promoters that are

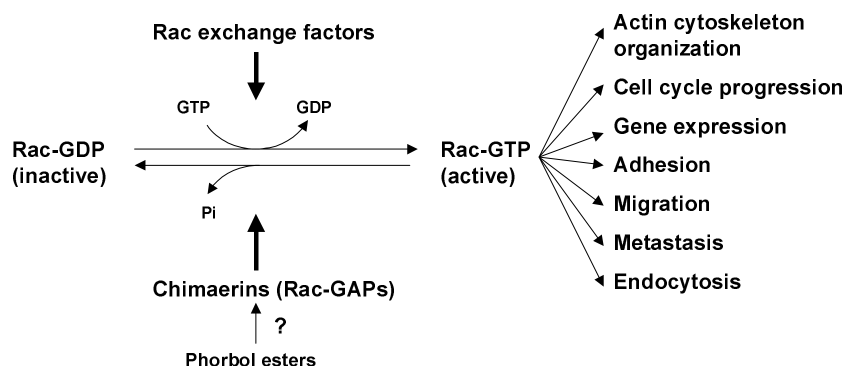


Fig. 2.– Chimaerins have GAP (GTPase activating protein) activity for the small GTP binding protein Rac. Rac cycles between an "on" and an "off" state. Rac exchange factors switch the "off" (GDP-bound) to the "on" (GTP-bound) state, leading to Rac activation. GAPs (such as chimaerins) accelerate GTP hydrolysis leading to Rac inactivation.

totally unrelated to PKC isozymes (Fig. 1). These proteins include chimaerins (a family of Rac GAPs), RasGRP (a Ras exchange factor), *Caenorhabditis elegans* Unc-13 and mammalian Munc13s (a family of proteins involved in exocytosis). An important feature of these novel phorbol ester receptors is that, unlike cPKCs and nPKCs, they have a single copy of the C1 domain. A second distinctive characteristic of these proteins is that they do not possess a kinase domain in their structure. These observations have important implications for the study of the biology of phorbol esters because they challenge the widely accepted dogma that all phorbol ester responses proceed through the activation of PKC isozymes.

Chimaerins: phorbol ester receptors that regulate the small GTP-binding protein Rac

Chimaerins are GTPase activating proteins (GAPs) for Rac, a small GTP-binding protein that plays a critical role in the regulation of actin cytoskeleton, cell cycle progression and malignant transformation. The first chimaerin isozyme, originally named n-chimaerin (and then renamed α1-chimaerin), resembles a "chimaera" between the regulatory domain of PKC and the GAP (GTPase-activating protein) domain of BCR, the Breakpoint Cluster Region protein involved in the translocation of Philadelphia chromosome in chronic myelogenous leukemia. Four chimaerin isoforms (α1- or n-, α2-, β1-, and β2-chimaerin) have been isolated to-date which are spliced variants from the α- and β-chimaerin genes. α2- and β2-chimaerins also possess a N-terminal putative SH2 domain, which may be involved in binding to phosphotyrosine proteins. The C1 domains in chimaerin isoforms possess approximately 40% homology to those in PKC isozymes. Our experiments revealed that α- and β-chimaerins bind phorbol esters and DAGs with affinities in the same low nanomolar range as cPKCs and nPKCs. Moreover, deletion of the C1 domain or mutation of essential cysteines completely abolished phorbol ester binding to chimaerins.

The biological role(s) of chimaerins have yet to be defined. Chimaerins possess GAP activity for the small GTP binding protein Rac, thereby accelerating the hydrolysis of GTP from this small GTP-binding protein, which leads to its inactivation. Using COS cells we have shown that phorbol esters and DAGs induce the subcellular redistribution or translocation of β2-chimaerin, suggesting the possibility of a positional regulation. A striking observation is that β2-chimaerin translocates to the perinuclear region upon phorbol ester or DAG treatment, thereby suggesting unique regulatory mechanisms and specialized functions which remain undetermined.

We have strong evidence that chimaerins regulate Rac activity and signaling in cellular models. Interestingly, overexpression of β2-chimaerin in COS cells results in reduced levels of GTP bound to Rac upon stimulation of receptors for epidermal growth factor (EGF). Using luciferase reporters for the transcription factor ATF2, we found that β2-chimaerin (or its GAP domain) inhibits muscarinic-induced activation of this luciferase reporter. This effect is mediated by the JNK (c-Jun N-terminal kinase) cascade, an event that is dependent on Rac.

Our prediction is that the chimaerin family of phorbol ester/DAG receptors plays an important regulatory role in Rac-mediated events, including actin cytoskeleton organization, cell cycle progression, malignant transformation, adhesion and migration. The contribution of chimaerins as mediators of phorbol ester effects in cellular models is a subject of intense investigation in our labora-

tory. As multidomain proteins, chimaerins may play a central role in signal transduction pathways involving DAG generation, small GTPases and tyrosine kinases.

Conclusion

For the last two decades the phorbol esters and related compounds have been the most common tools for the activation of PKC isozymes in cellular models. An important issue is that it is critical to dissect the biological roles of individual PKC isozymes in cellular models. The diversity of effects of PKC isozymes (including opposing roles in cell growth and apoptosis, and effects on differentiation) and the unique pharmacological properties of PKC ligands, make PKC isozymes an attractive target for selective pharmacological exploitation in cancer chemotherapy and other diseases.

A second important issue is that novel "non-kinase" phorbol ester receptors have been recently discovered. This challenges the use of phorbol esters as selective PKC activators. It is conceivable that some of the biological responses attributed to PKC may involve the activation of these novel receptors. It is of primary impor-tance to delineate the cellular functions and signaling pathways controlled by each phorbol ester receptor, determine their cellular targets, and establish their mechanisms of activation in cells. Rationale design of selective agonists and antagonists for each class of phorbol ester receptor is a key to decipher their function.

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INTERACTING SIGNALING PATHWAYS IN THE PATHOGENESIS OF SQUAMOUS CELL CARCINOMA

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Introduction

The elucidation of specific genetic changes associated with early events in human cancer pathogenesis has focused efforts to relate these changes to particular characteristics of the neoplastic phenotype. The mouse skin carcinogenesis model lends itself well to these analyses. Activating mutations of the ras gene family and upregulation of the epidermal growth factor receptor (EGFR) are frequently detected in human squamous cell carcinomas and mouse skin papillomas. Similarly, inactivation of the p53 tumor suppressor gene, interruption of the TGFβ signaling cascade and epigenetic changes in AP-1 regulated genes contribute to malignant progression in important human cancers and in experimental skin carcinogenesis. We have placed particular emphasis on defining how the biochemical consequences of these genetic alterations contribute to specific changes in the neoplastic phenotype.

The clearly defined genetic and phenotypic stages in the evolution of squamous tumor development on mouse skin provides an opportunity to evaluate mechanisms of carcinogenesis in a stage-specific manner. Initiation of skin carcinogenesis is manifested by the development of multiple benign tumors, squamous papillomas, each representing a clonal expansion of initiated cells. Papillomas differ from normal epidermis by three phenotypic markers: hyperproliferation, altered expression of keratinocyte genes, and defective terminal cell death. Therefore, to understand the genetic and biochemical basis for the initiated phenotype, one must elucidate the pathways that control these processes in normal keratinocytes and investigate how these pathways are altered in initiated cells. In previous studies we have established methods to maintain normal mouse and human keratinocytes *in vitro* and regulate specific states of epidermal maturation. We have developed and characterized *in vitro* models that recapitulate the genotypic and phenotypic alterations characteristic of cancer initiation *in vivo* and established procedures to analyze modified cells *in vivo* by skin grafting, defined markers characteristic of normal and neoplastic skin keratinocytes and generated reagents to monitor those markers.

The epidermal growth factor receptor and protein kinase C interact to control cell growth and cell death

The regulation of keratinocyte proliferation is under the control of multiple cytokines. We have focused on the epidermal growth factor receptor (EGFR) since it is constitutively activated in epidermal papillomas and frequently amplified in squamous cell carcinomas[1]. Furthermore, overexpression of TGFα by transgenic targeting to mouse epidermis followed by topical promotion with 12-O-tetradecanoylphorbol-13-acetate (TPA) or wounding is sufficient to produce squamous papillomas on mouse skin[2,3]. Keratinocytes express four members of the EGFR ligand family (TGFα, amphiregulin, beta-cellulin, and HB-EGF)[4], and the expression of all four ligands is increased in neoplastic keratinocytes. EGF and all four other EGFR ligands stimulate mouse keratinocyte proliferation *in vitro*[5]. Genetic deletion of the EGFR in mice results in early postnatal death and a hypoplastic epidermis with a low BrdU labeling index[6], suggesting this receptor is a positive growth regulator for the normal epidermis. However, hair follicle proliferation is not reduced in EGFR null skin, and abnormal wavy hair fibers are produced[6,7]. Remarkably, when EGFR null epidermis was grafted to nude mice to prolong the *in vivo* analysis, the grafted epidermis became hyperplastic, hair follicle proliferation and the aberrant hair phenotype were maintained, and an abnormal pattern of hair differentiation was revealed[7]. This was characterized by premature expression of the hair follicle differentiation markers, keratin 6, keratinocyte transgluta-

minase, and the hair keratins mHa2 and hacl-1. Furthermore, in skin grafts, the null epidermis, but not the wildtype epidermis, remained hyperplastic, suggesting that EGFR contributes a restorative function to the epidermis in a wound healing response. In null but not wildtype grafts, the hair follicles were eventually consumed in an inflammatory reaction. Together, these results indicate that the EGFR has complex functions in the skin, including the regulation of epidermal proliferation, proper timing of hair follicle differentiation, protection of hair follicles from immune/inflammatory reactions and the modification of the epidermal wound response. However, the EGFR is not essential for hair follicle proliferation.

The EGFR also participates in a keratinocyte differentiation and death pathway through an interaction with PKC signaling as first revealed by our studies on keratinocytes initiated *in vitro* by the introduction of a *v-ras* oncogene via a replication defective retrovirus[8]. Oncogenic ras is sufficient to initiate keratinocyte neoplasia, and initiated cells *in vitro* are resistant to calcium-induced terminal cell death[9]. This terminal phase of the differentiation program in normal keratinocytes is regulated by PKC as indicated by the use of PKC activators and inhibitors[10]. We found that *v-ras*-keratinocytes have increased activity of PKCα but reduced calcium-independent PKC activity[11]. While PKC δ, ε, or η were possible candidates for reduced activity, we discovered that PKCδ is tyrosine phosphorylated and catalytically inactivated in *v-ras*-keratinocytes[8]. Earlier studies had revealed that TGFα and aluminum fluoride together could mimic the resistance to calcium-induced terminal cell death caused by *ras* initiation *in vitro*[12], and we found that activation of the EGFR is the signal for tyrosine phosphorylation of PKCδ in initiated keratinocytes[13]. *In vitro* kinase reactions with recombinant PKCδ and EGFR, antibody depletion studies with solubolized keratinocyte membranes, and isolation of PKCδ from EGFR null keratinocytes, led to the conclusion that PKCδ was not a direct substrate for the activated EGFR. Instead, members of the Src kinase family, activated by EGFR, tyrosine phosphorylated and inactivated PKCδ[13]. This interaction of EGFR and PKCδ is not confined to neoplastic keratinocytes, as PKCδ is tyrosine phosphorylated in normal epidermis, and this is reduced in EGFR null keratinocytes[14]. Furthermore, PKCd tyrosine phosphorylation is detected only during terminal differentiation of normal keratinocytes and is associated with a 30 fold increase in cell-associated TGFα. Together these findings indicate that EGFR-mediated inactivation of PKCδ in terminal differentiation may be important physiologically in skin while inhibition of PKCδ in basal cells through ras stimulated EGFR activity may contribute to neoplastic development.

Direct evidence that PKCδ was involved in a keratinocyte death pathway came from studies utilizing a PKC δ adenovirus vector[15]. Introduction and activation of exogenous PKCδ in normal and neoplastic keratinocytes caused an apoptotic cell death program associated with caspase activation. In collaborative studies with Marcelo Kazanietz at the University of Pennsylvania, similar results were obtained with prostate cancer cells[16]. In keratinocytes, activated PKCδ translocates to mitochondria and alters mitochondrial membrane potential. A GFP-PKCδ fusion construct and a kinase-dead mutant GFP-PKCδ were transfected into keratinocytes to follow subcellular localization after TPA activation. After an initial translocation of both fusion proteins from cytosol to plasma membrane within a few minutes, the intact fusion protein, but not the kinase-dead mutant GFP-PKCδ, translocated to mitochondria. Thus, mitochondrial localization requires an active catalytic domain[15]. Since mitochondrial electron transport inhibitors prevented the lethal effects of PKCδ, it appears that an alteration in mitochondrial function mediates this keratinocyte death pathway.

A substantial reduction in PKCδ tyrosine phosphorylation was detected in *v-ras*-EGFR null-keratinocytes, yet these cells were able to form tumors when grafted to nude mice *in vivo*[5]. However, these tumors achieved only 1/5 the size of tumors formed from grafting v-ras-wildtype-keratinocytes from the same litters. *In vitro* analysis indicated that ras transduction increased proliferation of EGFR null keratinocytes, and conditioned medium from ras transformed keratinocytes also stimulated growth of keratinocytes lacking EGFR. Together, these results imply that other *ras* stimulated pathways and secreted factors could partially compensate for the absence of EGFR in neoplastic development. Both wildtype and EGFR null tumors had similar high rates of proliferation when examined by BrdU labeling and similar numbers of apoptotic cells when tested by TUNEL staining. However, the distribution of BrdU labeled cells was very different in tumors from the two genotypes. Null tumors had fewer BrdU labeled cells in the basal cell compartment and many more labeled cells in the suprabasal compartment.

BrdU pulse labeling of tumors formed from ν-*ras*-EGFR null-keratinocytes grafted to nude mice indicate a rapid migration of BrdU labeled tumor cells out of the proliferating basal cell compartment into the suprabasal compartment[17]. Double label analysis reveals that these cells undergo premature differentiation and cell cycle arrest. *In vitro*, ν-*ras*-EGFR null-keratinocytes migrate through extracellular matrix more rapidly and attach less readily to certain matrix components present in the basement membrane when compared to wildtype ras-initiated keratinocytes. Together these results indicate that the EGFR is essential for maintaining tumor cells in the proliferative pool. This may explain why the EGFR is frequently amplified or the ligands overexpressed in squamous tumors. We propose that, in the absence of

EGFR, another receptor-ligand pathway may predominate to maintain high tumor cell proliferative activity as seen in EGFR null tumors. It would follow that this compensatory growth stimulatory pathway is unable to prevent migration and premature differentiation of tumor cells, thus restricting tumor size.

Elucidation of pathways that contribute to premalignant progression and malignant conversion

Mutation and loss of heterozygosity of p53 or inactivation of p53 by premature degradation are frequently detected in squamous cell cancers of the skin, cervix, internal squamous organs and other cancers of lining epithelia. The vast majority of p53 mutations in tumors result in a protein that is transcriptionally inactive suggesting that this activity is critical for its action as a tumor suppressor. Two pathways impacted by transcriptional activity of this multifunctional protein are cell cycle control and apoptosis. Control of the cell cycle is at least in part related to the p53 mediated induction of $p21^{waf}$, an inhibitor of cyclin-dependent kinases and an important component of cell cycle checkpoints that control genomic stability. We found that keratinocytes from p53 null mice have reduced levels of p21waf protein[18]. Two lines of evidence indicate that reduction in $p21^{waf}$ is not responsible for the accelerated malignant conversion detected in p53 null skin papillomas. *v*-ras-$p21^{waf}$ null-keratinocytes are fully responsive to calcium induced growth inhibition and do not undergo accelerated malignant conversion when grafted to nude mice, opposite to results obtained with *v*-ras-p53 null-keratinocytes[19]. Chemically-induced papillomas on $p21^{waf}$ null mice have the same frequency of malignant conversion as wildtype littermates, and in contrast to p53 null mice, the papilloma incidence in $p21^{waf}$ mice increased almost 3 fold over wildtype controls[20].

The induction of apoptosis by p53 is complex and, in part, related to upregulation of the proapoptotic protein Bax in some cell types and several novel recently discovered genes in other cell types [21-23]. Deletion of p53 enhances the establishment of immortal keratinocyte cell lines [24] suggesting p53 could participate in keratinocyte mortality. Furthermore, p53 transcriptional activity increases in association with keratinocyte terminal differentiation[25], and p53 null tumors are highly undifferentiated. We conducted a differential display comparative analysis of mRNA sequences from differentiating p53 null and wildtype keratinocytes in order to detect p53 regulated genes involved in differentiation or mortality. Among the confirmed differences, we cloned mtCLIC, a novel gene that encodes a 28kDa chloride channel protein localized in the cytoplasm and mitochondria (26). mtCLIC is a new member of a small family of organellular chloride channel proteins, is expressed in all tissues examined, reduced but not absent in tissues and keratinocytes from p53 null mice, and detected as an EST in the two cell embryo. mtCLIC is upregulated by p53 and TNFα in keratinocytes and increases in differentiating cells in the presence or absence of p53. mtCLIC transcripts and protein are induced within 6 hours of exogenous expression of p53 in keratinocytes.

The discovery of mtCLIC and its regulation by p53 and TNFα, has directed our attention to define the cellular functions and regulation of this protein, to understand its potential contribution to p53 activity in normal and tumor cells, and to determine its relevance to human cancer. mtCLIC is similar to proapoptic Bax in that both have cytoplasmic and mitochondrial locations, two transmembrane domains, putative ion channel activity and p53 regulation. We have generated a series of reagents for mtCLIC analysis including GFP-mtCLIC and mtCLIC-GFP fusion constructs, monospecific polyclonal antibodies to unique C and N terminal peptides, CMV-driven full length antisense orientation vectors, and mouse and human keratinocytes that are deficient or intact for p53. While mtCLIC is expressed in all organs tested, the levels are highest in heart, kidney, lung, liver and skin, and the basal levels are p53 dependent. Preliminary studies implicate mtCLIC as an important component of the cellular apoptotic response, both dependent and independent of p53, and loss of mtCLIC may be essential for tumor progression.

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