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We are investigating growth factor signaling in the mammary gland through the analysis of *Cripto*, a gene that is distantly related to epidermal growth factor (EGF). Previous studies have suggested that *Cripto* may play an autocrine or paracrine role in human breast, colorectal, gastric, and pancreatic carcinomas. To assess whether *Cripto* plays a role in normal breast development and whether it has a causal role in tumorigenesis, we have generated retroviruses and transgene constructs to examine the consequences of ectopic expression in cell culture and in transgenic mice. To analyze receptor binding by CRIPTO protein and to initiate the molecular cloning of the CRIPTO receptor(s), we have generated histochemical affinity reagents to assay binding to cell-surface receptors. Using these reagents, we have demonstrated quantitative receptor binding of CRIPTO to mammary cell lines, and have observed a highly localized distribution of CRIPTO binding sites to tissue sections during early mouse development. These results demonstrate the existence of high-affinity CRIPTO receptors and represent the groundwork for their molecular identification.

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FOREWORD

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Michael McShan 10/21/97
PI - Signature Date

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5. Introduction:

5.a. Background and significance

5.a.(i) Cripto *encodes a signaling factor that is overexpressed in breast cancer*

Our research on breast development and tumorigenesis is focused on investigating the molecular properties of *Cripto,* a gene that encodes a secreted growth factor-like molecule that is distantly related to epidermal growth factor (EGF). Previous studies have implicated *Cripto* as a dominant oncogene in human breast, colorectal, gastric, and pancreatic carcinomas [1-5]. These investigations have consistently found activation of *Cripto* expression in primary tumors arising from tissues where the gene is normally dormant. For example, a survey of 68 primary ductal and lobular breast carcinomas demonstrated that *Cripto* is expressed in approximately 80% of the tumors examined, but in only a small proportion (about 10%) of adjacent noninvolved mammary samples, and at very low levels [5]. In addition, these correlative findings are supported by *in vitro* transformation studies which show that overexpression of *Cripto* is able to induce focus formation by NIH/3T3 cells [6] and soft agar colony formation by NOG-8 mouse mammary epithelial cells [7]. Taken together, these studies are consistent with an autocrine or paracrine role for *Cripto* in breast carcinoma growth.

Other investigations have provided evidence for a role of *Cripto* activation in colorectal tumorigenesis. Two studies have found that a majority (60-80%) of primary human colorectal carcinomas express *Cripto,* as opposed to only a small fraction (about 5%) of noninvolved colon samples [1, 3]. Examination of a small group of adenomas showed that significantly more tubulovillous adenomas expressed *Cripto* than did tubular adenomas, suggesting that *cripto* expression might be associated with colorectal tumor progression [3]. An autocrine or paracrine role for *Cripto* is supported by the observation that GEO and CBS colon cancer cells show greatly reduced growth rates and tumorigenicity *in vitro* and *in vivo* after the inhibition of endogenous *Cripto* expression by antisense oligonucleotides [8]. Thus, these lines of evidence suggest that *Cripto* overexpression is a frequent event in human breast and colon tumorigenesis.

5.a.(ii) Cripto is a member of a novel family of signaling molecules

We became interested in *Cripto* through our studies of mesoderm formation during murine development, in which we isolated a novel related gene that we named *Cryptic* [9]. Our analysis of sequence alignments indicated that *Cripto, Cryptic,* and a *Xenopus* gene named *FRL-1* [10] define a new family of EGF-related genes, which we named the "CFC" (*Cripto, Frl-1,* and *Cryptic*) family (Fig. 2). The putative protein products of these genes share a potential N-terminal leader sequence, a variant EGF-like motif, a novel conserved cysteine-rich region (the CFC motif), and a C-terminal hydrophobic domain, which may represent a transmembrane region. At the amino acid level, however, most of the sequence similarity occurs in the single EGF-like motif and the CFC motif. Interestingly, the EGF-like motifs of these genes have characteristic features that differ from other EGF motifs, in that the first two cysteines are adjacent, and the spacing between the third and fourth cysteines is reduced. Despite their unusual sequence features, these variant motifs appear to represent *bona fide* EGF motifs, since several key residues that are found in all EGF motifs are conserved in CFC family members. Furthermore, molecular modeling of the CRIPTO EGF-like motif based on the structures of murine EGF, human TGF-α, and human clotting factor IX suggests that it adopts a typical EGF-like fold [11].

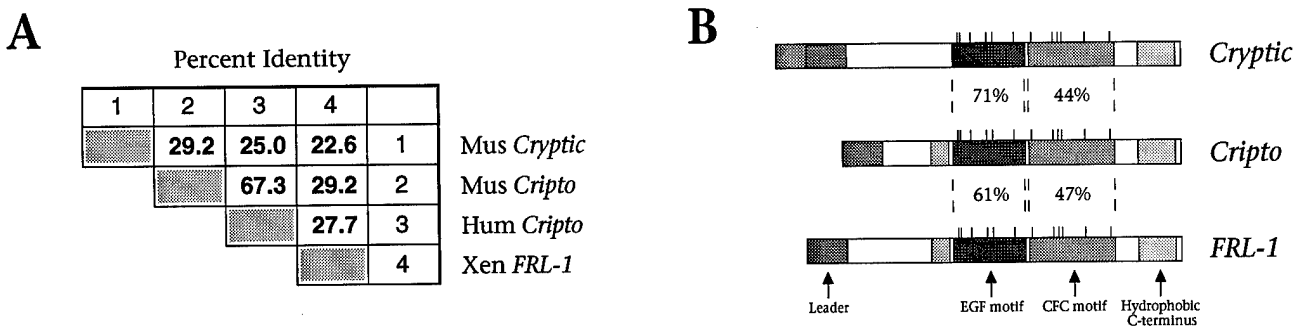

Figure 1. Sequence relationships of members of the CFC family. *(A)* Similarity of family members, given by the percentage of identical amino acid residues, based on the alignment in [9]. The distant relationship of *FRL-1* to *Cryptic* and *Cripto* indicates that *FRL-1* is not an ortholog of either murine gene. *(B)* Schematic alignment of the *Cryptic, Cripto,* and *FRL-1* encoded proteins, drawn approximately to scale [9]. The percentage amino acid identity of murine *Cryptic* and *FRL-1* with murine *Cripto* in the EGF-like motif and a novel cysteine-rich repeat (CFC motif) is indicated, together with the locations of conserved cysteines (vertical lines); no significant conservation occurs elsewhere, apart from a small region of similarity between *Cripto* and *FRL-1* upstream of the EGF motif (light gray box). For *Cryptic,* the two boxes shown for the leader peptide correspond to the two in-frame translation initiation sites [9].

Several studies have indicated that the protein encoded by the human *Cripto* gene possesses potent growth factor activity. First, mitogenic activity has been demonstrated in experiments that used human CRIPTO protein secreted by transfected CHO cells, or a refolded 47-mer peptide that contains the CRIPTO EGF-like motif [12]. Both the CHO conditioned medium and the refolded peptide stimulate the proliferation of several human breast carcinoma cell lines, including MDA-MB-453 and SK-BR-3, as well as the untransformed mammary epithelial cell line 184A1N4 [12]. Secondly, refolded CRIPTO peptide can bind to murine HC-11 mammary epithelial cells and induce tyrosine phosphorylation of the SH2-adaptor protein Shc, resulting in increased association of Shc with Grb2 and elevated MAP kinase activity, indicating activation of components of the *ras* signaling pathway [13]. Combined with studies that have documented *Cripto* transforming activity [6, 7] and *Cripto* overexpression in human breast, colorectal, gastric, and pancreatic carcinomas [1, 3, 4, 14, 15], these findings suggest that *Cripto* may be involved in autocrine or paracrine signaling during tumorigenesis.

5.a.(iii) The Cripto receptor(s) is not yet identified

Recent observations suggest that CRIPTO, and by extension other members of the CFC family, binds to an as yet unidentified cell-surface receptor(s). In particular, the available evidence indicates that CRIPTO, unlike other EGF-related growth factors, interacts with a receptor(s) that is distinct from the four known members of the *erbB* receptor family (EGF receptor, c-*erbB-2/neu/HER-2,* c-*erbB-3,* and c-*erbB-4).* First, the variant EGF-like motif found in members of the CFC family lacks important residues that mediate high-affinity binding to *erbB* receptors, including the residues of the missing "A loop" and the conserved arginine residue prior to the sixth cysteine ([11, 16]). Secondly, the high-affinity binding of ^{125}I-labeled refolded CRIPTO peptide to HC-11 cells is not competed by *erbB* receptor ligands such as EGF, TGF-α, amphiregulin, or heregulin β1 [13]. Finally, the refolded CRIPTO peptide does not stimulate receptor tyrosine phosphorylation in Ba/F3 cells stably transfected with single *erbB* receptor genes or with pair-wise combinations [13].

With regard to potential receptor interactions of CFC family members, it is particularly noteworthy that *FRL-1* was isolated on the basis of its ability to induce FGF receptor

autophosphorylation in a heterologous yeast expression system [10]. Similar to secreted forms of FGF, *FRL-1* possesses mesoderm and neural-inducing activities in *Xenopus* animal cap assays, which can be blocked by expression of a truncated FGF receptor that acts in a dominant-negative manner [10, 17, 18]. At present, the molecular basis for the interaction of FRL-1 with FGF receptor signaling is poorly understood. Although it has been proposed that FRL-1 represents a novel ligand for FGF receptors, there is no evidence that FRL-1 protein can directly bind to FGF receptors [10]. Alternatively, it is possible that FRL-1 indirectly activates the FGF receptor after binding to a novel receptor molecule, as has been found for activation of the EGF receptor by ligands for G-protein-coupled receptors [19]. Notably, recent studies have demonstrated other examples of FGF receptor signaling that appear to be mediated by non-FGF ligands [20, 21]. Regardless of the molecular mechanism involved, the sequence similarities of CRIPTO and CRYPTIC to FRL-1 suggest that these related CFC family members may also signal through FGF receptors. Elucidation of the molecular mechanisms involved in these potential signaling interactions will require the cloning of the cell-surface receptor(s) for CRIPTO.

5.b. Scope and purpose of research

At present, the evidence implicating *Cripto* in oncogenesis is indirect, and no causal role or molecular mechanism for *Cripto* in tumorigenesis has been delineated. Moreover, it is unclear whether *Cripto* may also represent a signaling factor involved in normal regulation of proliferation and differentiation of the mammary gland. Since perturbation of normal processes of growth and differentiation often represent primary events in oncogenic transformation, the investigation of *Cripto* may result in important insights for breast development and tumor progression.

To evaluate the putative oncogenic role of *Cripto,* we are utilizing *in vivo* and cell culture systems to examine mammary development and tumorigenesis, and are undertaking the molecular cloning of the *Cripto* receptor(s). First, we are generating *Cripto*-expressing transgenic mice to assay mammary tumorigenesis in a physiological system (*Technical Objective I*). Secondly, we have produced expression vectors that will allow us to examine the consequences of *Cripto* overexpression in cell lines that represent model systems for mammary epithelial differentiation and for human breast tumor progression (*Technical Objective II).* Finally, we have developed reagents and assays that will allow us to define the mammary cell types that express *Cripto* receptors and will facilitate the molecular cloning of the *Cripto* receptor(s) *(Technical Objective III).* These studies will permit the future biochemical investigation of downstream signaling events and the cellular and molecular consequences of *Cripto* overexpression.

6. Body:

6.a. Materials and methods

6.a.(i) Generation of transgenic mice

To produce transgenic founder animals, we are performing oocyte microinjection using standard techniques [22]. Briefly, linearized DNA constructs are microinjected into oocyte pronuclei, followed by surgical re-implantation into the oviducts of pseudopregnant females. To assay the genotype of the resulting offspring, tail DNA is analyzed for transgene incorporation by Southern blotting. We will generate stable lines for each transgene construct in the FVB/N inbred strain of mice, which we have used previously for ectopic expression experiments [23]. This inbred strain features superior reproductive performance and prominent oocyte pronuclei that facilitate

DNA microinjection [24], and has been used extensively in studies of mammary tumorigenesis [25, 26].

All mice are maintained under specific pathogen-free (SPF) conditions, using micro-isolator cages, HEPA-filter cage racks, and laminar flow changing hoods. Our research with laboratory mice is covered under animal use protocols that have been approved by the Institutional Animal Care and Use Committee (IACUC) of UMDNJ - Robert Wood Johnson Medical School. These experiments are covered under protocols I94-072, I95-009, and I95-010, which were re-approved on 11/16/96, 5/27/97, and 5/27/97, respectively. Institutional approval guarantees compliance with Public Health Service policies for the humane care and use of laboratory animals.

6.a.(ii) Production of retroviruses for Cripto *expression*

To produce retroviruses that express *Cripto,* we transiently transfect Phoenix A amphotropic packaging cells with *LZRS-pBMN-2* vectors that contain appropriate inserts [27]. To obtain highly efficient transfection, packaging cells are transfected in serum-free Opti-Mem medium using Lipofectamine (Gibco). Following transfection, culture supernatants containing retroviral particles are harvested after two days. Target cells such as MCF-10A or HC-11 are infected by incubation with viral supernatants for five hours in the presence of 4 μg/ml polybrene, as described [28].

6.a.(iii) Alkaline phosphatase fusion proteins for receptor binding studies

To produce histochemical affinity reagents for receptor binding experiments, we have produced constructs to express fusion proteins of CRIPTO with human placental alkaline phosphatase in mammalian cells. For expression of these alkaline phosphatase (AP) fusion proteins, COS-7 cells are transiently transfected using Lipofectamine (Gibco), and conditioned media collected three days later for analysis of alkaline phosphatase activity. These cell supernatants are screened in a colorimetric microplate assay for AP activity by measuring the hydrolysis of *p*-nitrophenyl phosphate, with the amount of AP activity defined as the maximum rate of change of $O.D._{405}$/hour under standardized assay conditions [29]. Quantitative cell binding assays are performed by incubating cells with CRIPTO-AP containing supernatants, or AP containing supernatants as a negative control. After washing and lysis of the cells, nuclei are spun down and the remaining supernatant heated to inactivate endogenous alkaline phosphatase activity (but not that of the heat-stable fusion protein), followed by assaying for *p*-nitrophenyl phosphate hydrolysis [30]. Scatchard analysis is based on 30 units of AP activity corresponding to 1 pmol of AP fusion protein in this assay [29].

To assay AP fusion protein binding to tissue sections, frozen sections (12 μm) of embryonic tissue are overlaid with supernatants from COS cells transfected with *AP4/mCripto* or the parental *APtag-4* vector. The sections are then fixed, heated to destroy endogenous alkaline phosphatase activity, and stained with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP), essentially as described [31]. For our experiments, the COS cell supernatants obtained by transfection of *AP4/mCripto* or the *APtag-4* control have similar AP activities. No binding is ever detected for the control.

6.b. Results and discussion

6.b.(i) Technical Objective I: Generation of Cripto *transgenic mice*

Rationale: To investigate the effects of *Cripto* on mammary development and oncogenesis, we are producing transgenic mice that overexpress *Cripto* in their mammary glands. This methodology has several advantages because transgenic mice can represent model systems for *(i)*

assessment of the transforming potential of candidate oncogenes in a physiologically-relevant environment, *(ii)* study of the multiple stages of tumor progression, and *(iii)* investigation of the efficacy of potential therapies. Notably, transgenic approaches can assess the influence of epigenetic factors such as hormonal levels and epithelial-stromal interactions, which are known to be significant in human breast cancer.

Since we presume that CRIPTO acts as an secreted signaling molecule, the biological activity of a *Cripto* transgene should depend upon the levels of protein secretion attained *in vivo*. Therefore, the efficiency of CRIPTO protein secretion represents a primary consideration for the design of transgene constructs. This issue is particularly noteworthy because the human *Cripto* gene, unlike mouse *Cripto*, is believed to lack a signal sequence to direct secretion from cells [6, 32]. Moreover, our recent studies have shown that CFC proteins, including CRIPTO, are poorly secreted from transfected cells in culture, probably because the endogenous signal sequence is non-conventional and directs inefficient secretion ([9] and data not shown). In contrast, when we replace the endogenous murine *Cripto* signal sequence with a highly efficient signal sequence, we obtain ten-fold higher levels of CRIPTO secretion from transfected cells. Therefore, an unmodified *Cripto* transgene may not be sufficient to induce a strong and reproducible phenotype in transgenic mice. Consequently, we have constructed transgene expression vectors containing either an unmodified (wild-type) *Cripto* gene or a modified *Cripto* gene that should direct high-level protein secretion.

Results: We are using the mouse mammary tumor virus (MMTV) promoter to direct expression of CRIPTO transgenes to the mammary epithelium of transgenic mice. For this purpose, we are utilizing the expression vector *MMTV-SVPA* [33], which has an MMTV long terminal repeat (LTR) enhancer/promoter, a cloning site, and SV40 polyadenylation sequences (Figure 2A). We have subcloned mouse and human *Cripto* cDNAs into this vector, both in unmodified and modified (Sec-*Cripto*) forms, for a total of four distinct constructs (Figure 2B). At present, we have injected the murine and human *Cripto* transgene constructs containing the unmodified leader peptides, and have recently obtained progeny mice that are being genotyped.

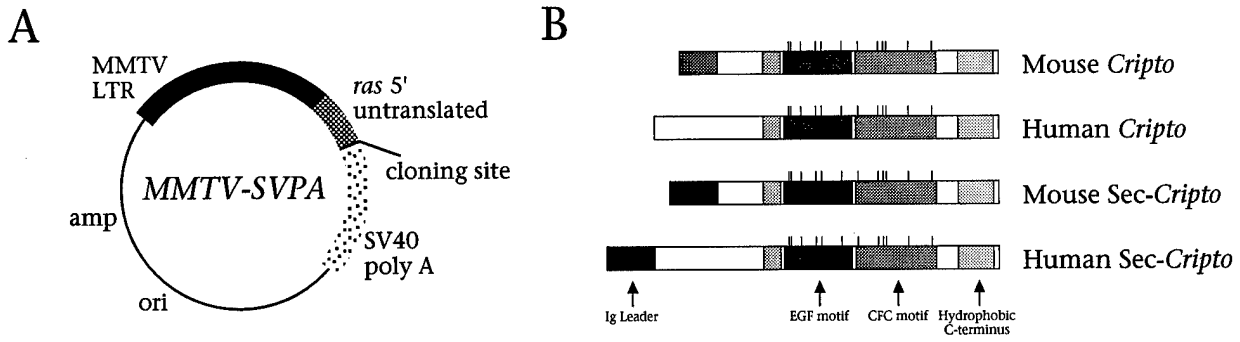

Figure 2. Schematic representation of the transgene vector and inserts. ***(A)*** **The *MMTV-SVPA* expression vector** contains the long terminal repeat from the mouse mammary tumor virus, 5' untranslated sequences from *ras*, and the 3' polyadenylation region from SV40 [33]. ***(B)*** **The mouse and human *Cripto* transgene inserts** have been produced in unmodifed and modified forms (Sec-*Cripto*). Note that the unmodified human CRIPTO protein is slightly larger than its mouse homologue and lacks a putative leader peptide (dark gray box). For the modified expression constructs, we have utilized the leader peptide from a murine immunoglobulin kappa chain gene (derived from the *pSecTagB* expression vector; Invitrogen) as a replacement for the endogenous murine CRIPTO leader peptide or as a fusion with the entire human CRIPTO sequence.

6.b.(ii) Technical Objective II: Expression of Cripto *in cell culture model systems*

Rationale: To examine the role of *Cripto* in cellular proliferation, differentiation and transformation, we have developed expression constructs that will enable us to manipulate the expression of *Cripto* in relevant cell types. For this purpose, we are utilizing retroviral gene transfer as a strategy to efficiently transfect *Cripto* into cells. Notably, many recent advances have made retroviral gene transfer a powerful approach for the rapid and efficient introduction of genes into mammalian cells (*e.g.,* [27, 34]). In particular, the development of efficient packaging cell lines has markedly reduced the time and effort required to produce high-titer virus.

The general strategy for retroviral gene transfer is diagrammed in Figure 3. As shown, the gene of interest (*Cripto* or a *lacZ* control) is cloned into a suitable replication-incompetent retrovirus vector. The vector is transfected into a packaging cell line that supplies the proteins required to make a viral capsid *in trans*. The virus particle is released into the supernatant where it can be collected and used to infect target cells, through interaction of the viral particle with cell surface receptors. Use of an amphotropic packaging line results in production of viruses that can be utilized to infect a variety of mammalian cells, including human and rodent cells.

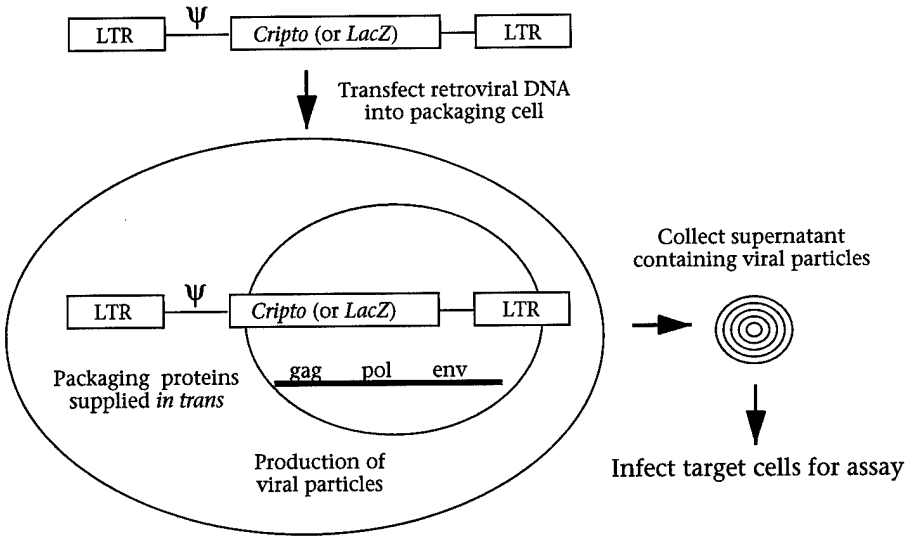

Figure 3. Strategy for production of retroviral expression vectors. Details of the general strategy for construction, production, and analysis of retroviruses are described in [28].

Results: For our studies, we are using a retroviral vector (*LZRS-pBMN-2*) developed by Gary Nolan and colleagues [27]. This vector has the following salient features for efficient gene expression and high level virus production: *(i)* full-length Moloney LTRs for efficient gene expression, *(ii)* extended Psi (ψ) sites to direct appropriate packaging of the mRNA, *(iii)* a selectable marker (the puromyocin-resistance gene) that allows for enrichment of cells containing the retroviral vector, and *(iv)* Epstein-Barr virus origin and nuclear retention sequences, which maintain the retroviral vector as an episome. We have utilized the Phoenix packaging cell line, which is a modified 293T cell line derivative that is available for both ecotropic and amphotropic viral production [27], and have found that high-titer virus (~10^7) can be obtained from supernatants following transient transfection.

We have generated *LZRS-pBMN-2* vectors containing mouse and human *Cripto* inserts, using either unmodified or modified signal sequences (Figure 2B). In current studies, we have

generated retroviral supernatants corresponding to each of these expression vectors. In preliminary experiments using a control *LacZ* insert, we have obtained high levels of infection of target NIH/3T3 fibroblast cells (70 - 80%), as judged by β-galactosidase staining. In ongoing experiments, we are assaying the levels of protein production following infection of NIH/3T3 cells, using Western blotting with an anti-CRIPTO antiserum that we have generated (in the course of studies unrelated to this grant proposal).

6. b.(iii) Technical Objective III: Analysis of CRIPTO binding to cell-surface receptor(s)

Rationale: To develop methods to identify the *Cripto* receptor(s), we have used a histochemical affinity approach to investigate CRIPTO protein binding to putative cell-surface receptors. For this purpose, we have generated a soluble receptor affinity reagent that consists of CRIPTO protein fused to secreted human placental alkaline phosphatase (AP), which is expressed in transiently transfected COS cell supernatants [29-31]. The COS cell supernatants containing CRIPTO-AP fusion protein can then be used directly to visualize receptor binding on cell lines and tissue sections by histochemical staining [29-31]. This AP fusion protein approach provides a simple non-radioactive detection method that has been used to visualize ligand-receptor interactions in a wide range of studies (*e.g.*, [35, 36]). Other advantages of this methodology include its sensitivity, which is comparable to that obtainable by ^{125}I-labelled protein [35], and its histological resolution at the near-cellular level (*e.g.*, [31]). In our investigatations, we have used this methodology to demonstrate *(i)* quantitative binding of CRIPTO-AP fusion proteins to cultured cell lines, and *(ii)* specific patterns of binding to embryonic sections.

Results: ***(i) Quantitative binding:*** To investigate receptor binding and to assess the feasibility of this approach, we have generated fusion proteins of murine and human *Cripto* with secreted placental alkaline phosphatase using the *APtag-4* expression vector (Fig. 4A) [31], and have expressed these fusion proteins in culture supernatants of transfected COS cells. We have used the *APtag-4* vector for this purpose because it utilizes the endogenous alkaline phosphatase leader peptide to direct efficient secretion of fusion proteins, thereby avoiding potential difficulties with the CRIPTO leader peptide. For our initial studies, we have chosen to examine binding of human CRIPTO-AP to the mammary epithelial cell lines MDA-MB-453 and SK-BR-3, since these cell lines can be stimulated to proliferate by a refolded 47-mer CRIPTO peptide [12].

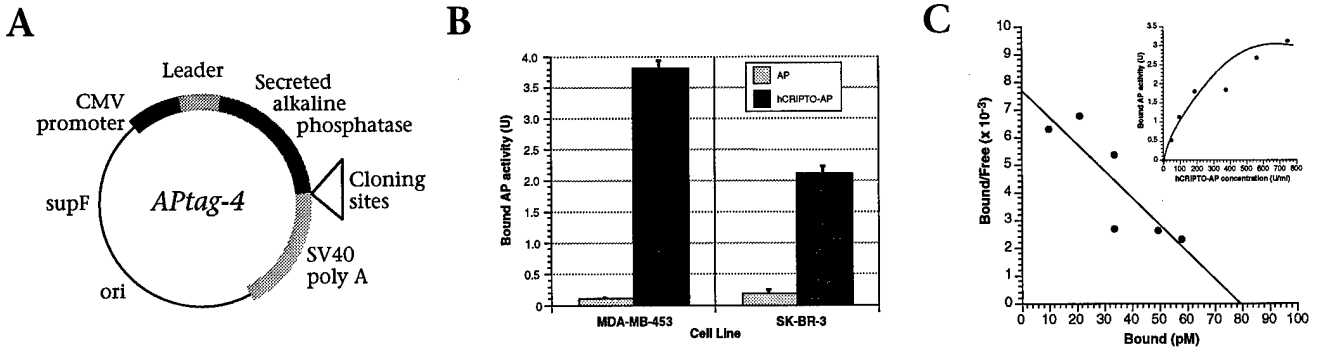

Figure 4. Binding of CRIPTO-AP fusion protein to cultured cells. ***(A)*** **Schematic map of the *APtag-4* expression vector.** A cytomegalovirus enhancer/promoter directs high-level expression of an intact secreted placental alkaline phosphatase gene that contains a polylinker cloning region for in-frame fusions at its 3' end. ***(B)*** **Quantitative binding of human CRIPTO-AP to mammary epithelial cell lines.** Parallel binding assays to MDA-MB-453 and SK-BR-3 cells were performed with CRIPTO-AP containing supernatants generated by transient transfection of *AP4/hCripto* and with unfused AP containing supernatants by

transfection of the *APtag-4* parental expression vector. The supernatants were adjusted prior to binding to have the identical concentration of AP activity (750 U/ml); each assay was performed in triplicate. *(C)* **Scatchard analysis of CRIPTO-AP binding to MDA-MB-453 cells.** Quantitative binding of human CRIPTO-AP to MDA-MB-453 cells was performed using dilutions of a CRIPTO-AP containing COS cell supernatant, with each concentration assayed in triplicate (primary data is shown in inset).

Our results show that human CRIPTO-AP fusion protein binds specifically to both the MDA-MB-453 and SK-BR-3 mammary cell lines (Fig. 4B), and displays saturable binding consistent with high-affinity interactions with cell-surface receptors (Fig. 4C). Scatchard analysis of this data indicates a K_d of 10 nM for CRIPTO-AP binding to MDA-MB-453 cells, with approximately 2.4 x 10^5 receptors present per cell. These figures are comparable to the recently published figures of K_d of 80 nM and 4.4 x 10^5 receptors for binding to MDA-MB-453 cells [13]. However, we note that this published binding data [13] has utilized an ^{125}I-labeled refolded synthetic peptide whose binding properties may not completely correspond to those of a full-length CRIPTO protein expressed in mammalian cells.

(ii) Distribution of binding sites in embryos: A particular advantage of the AP fusion protein approach is that it allows the visualization of receptor binding in tissue sections with near-cellular resolution [31]. Therefore, in our initial experiments we have examined the pattern of murine CRIPTO-AP binding to cryosections from mouse embryos at day 7.5 through 10.5 of gestation. We have found that CRIPTO-AP fusion protein binds broadly to the ectoderm and mesoderm during gastrulation, but later is localized to specific regions during organogenesis, predominantly to cell populations deriving from the cranial and trunk neural crest (Fig. 5). Overall, the distribution of CRIPTO-AP binding sites overlaps with the pattern of *Cripto* and *Cryptic* expression [9, 32, 37], such that each known site of expression of these genes correlates with the distribution of putative receptors for CRIPTO, and possibly CRYPTIC. Interestingly, CRIPTO-AP binding is also observed in several tissues where neither gene is expressed, raising the possibility that this binding may correspond to the expression of other *CFC* family members.

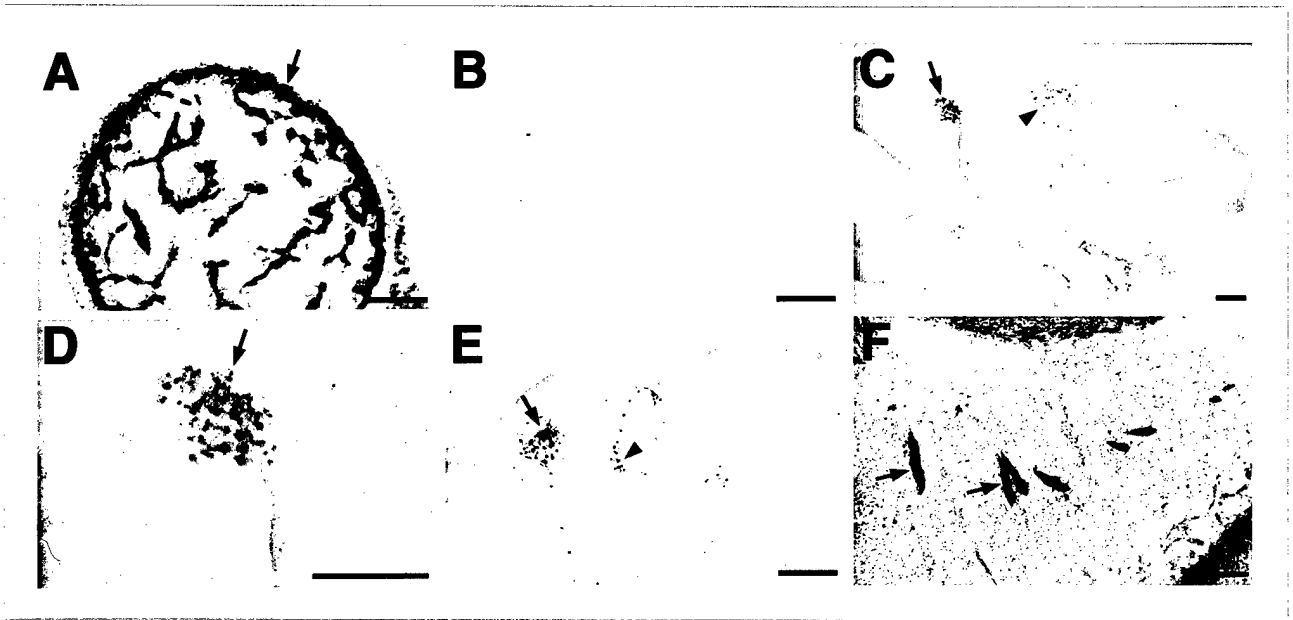

Figure 5. Binding of CRIPTO-AP fusion protein to embryonic tissue sections. *(A)* Transverse section through the ventricle of a day 9.5 embryo, showing CRIPTO-AP binding to myocardial cells (arrow), but not to endocardial cells (white arrowhead) or blood. *(B)* Lack of binding observed on a control section

adjacent to that in panel B, using culture supernatant from COS cells transfected with the parental *APtag-4* expression vector. *(C)* Transverse section through the head of a day 9.5 embryo, with CRIPTO-AP binding in the developing optic stalk (arrow), in cranial neural crest cells (arrowhead), and in the first branchial cleft (white arrowhead). *(D)* High-power view of the optic stalk from panel D. *(E)* Transverse section through a day 10.5 embryo, showing CRIPTO-AP binding to trigeminal neural crest tissue (arrow) and the first branchial cleft (arrowhead). *(F)* Sagittal section through the trunk at day 10.5, showing binding to spinal nerve fibers (arrows) that arise from the dorsal root ganglia. Scale bars correspond to 0.1 mm.

6.c. Relationship to Statement of Work:

As described above, we have made significant progress towards all three of the Technical Objectives proposed in our original grant application. In particular, we intend to adhere to the general time-table presented in the Statement of Work, as discussed below.

For *Technical Objective I,* we have generated all of the constructs that will be used for production of transgenic mice expressing mouse and human *Cripto* in the mammary gland. We are now in the process of injecting these constructs to obtain founder transgenic mice. Moreover, in the past year, we have successfully established our own microinjection facility for the production of transgenic mice, and have hired a technician with substantial experience in this technique to perform the microinjections and to manage our mouse colony. Therefore, we anticipate no significant difficulties in generating the proposed lines of transgenic mice *(Task 1),* and in pursuing the initial analysis of their phenotype *(Tasks 2, 3).* These studies should be invaluable in assessing the effects of *Cripto* overexpression upon mammary gland development and tumorigenesis *in vivo.*

For *Technical Objective II,* we have established the retroviral expression methodology in our laboratory, and have generated retroviruses expressing mouse and human *Cripto.* Using these retroviruses, we will now perform two lines of experiments to examine the effects of *Cripto* overexpression: *(i)* infection of HC-11 mammary epithelial cells to examine the effects of CRIPTO expression upon differentiation in culture; and *(ii)* infection of MCF-10A human mammary epithelial cells with these retroviruses to assay the effects of CRIPTO expression upon tumorigenesis in cell culture and in *nude* mice *(Tasks 8, 9).* Because our retroviruses can be used to infect a wide range of cell types, we have significantly expanded these studies beyond those that we originally proposed (which only considered MCF-10A cells; *Tasks 8-12*). Our studies should result in a detailed analysis of the effects of *Cripto* expression upon mammary differentiation and tumorigenesis in these cell culture model systems.

For *Technical Objective III,* we have sucessfully generated alkaline phosphatase fusion proteins to examine CRIPTO binding to cell lines and to tissue sections from mouse embryos. Thus, we have completed *Task 13,* and are nearing completion of *Tasks 14* and *15.* In current experiments, we are investigating the distribution of binding sites for CRIPTO-AP fusion proteins in mammary tissue *(Task 16).* Moreover, we have already initiated the construction of cDNA expression libraries from MDA-MB-453 cells to screen for clones that bind CRIPTO-AP fusion protein *(Task 17).* Thus, we have made substantial progress towards the analysis and future identification of the *Cripto* receptor(s).

7. Conclusions:

Over the past year, we have generated expression constructs and retroviral vectors for the analysis of the consequences of ectopic expression of *Cripto* in transgenic mice and in cell culture. Furthermore, we have generated histochemical affinity reagents to assay binding of CRIPTO to cell-surface receptors, and have successfully demonstrated binding to cell lines and tissue sections. Our results show that specific receptor(s) for CRIPTO exist on mammary cell lines that display high-affinity saturable binding. Moreover, we have found the distribution of binding sites for CRIPTO in mouse embryos is highly localized and is consistent with a putative receptor interaction. Finally, our analysis demonstrates the feasibility of this approach for the molecular cloning of the CRIPTO receptor(s), which represents a primary focus of our ongoing research.

8. References:

1. Ciardiello, F., Kim, N., Saeki, T., Dono, R., Persico, M.G., Plowman, G.D., Garrigues, J., Radke, S., Todaro, G.J., and Salomon, D.S. (1991). Differential expression of epidermal growth factor-related proteins in human colorectal tumors. Proc. Natl. Acad. Sci. USA *88,* 7792-7796.
2. Kuniyasu, H., Yoshida, K., Yokozaki, H., Yasui, W., Ito, H., Toge, T., Ciardiello, F., Persico, M.G., Saeki, T., Salomon, D.S., and Tahara, E. (1991). Expression of *cripto,* a novel gene of the epidermal growth factor family, in human gastrointestinal carcinomas. Jpn. J. Cancer Res. *82,* 969-973.
3. Saeki, T., Stromberg, K., Qi, C.F., Gullick, W.J., Tahara, E., Normanno, N., Ciardiello, F., Kenney, N., Johnson, G.R., and Salomon, D.S. (1992). Differential immunohistochemical detection of amphiregulin and cripto in human normal colon and colorectal tumors. Cancer Res. *52,* 3467-3473.
4. Friess, H., Yamanaka, Y., Buchler, M., Kobrin, M.S., Tahara, E., and Korc, M. (1994). Cripto, a member of the epidermal growth factor family, is over-expressed in human pancreatic cancer and chronic pancreatitis. Int. J. Cancer *56,* 668-674.
5. Qi, C.F., Liscia, D.S., Normanno, N., Merlo, G., Johnson, G.R., Gullick, W.J., Ciardiello, F., Saeki, T., Brandt, R., Kim, N., Kenney, N., and Salomon, D.S. (1994). Expression of transforming growth factor alpha, amphiregulin and cripto-1 in human breast carcinomas. Br. J. Cancer *69,* 903-910.
6. Ciccodicola, A., Dono, R., Obici, S., Simeone, A., Zollo, M., and Persico, M.G. (1989). Molecular characterization of a gene of the "EGF family" expressed in undifferentiated human NTERA2 teratocarcinoma cells. EMBO J. *8,* 1987-1991.
7. Ciardiello, F., Dono, R., Kim, N., Persico, M.G., and Salomon, D.S. (1991). Expression of cripto, a novel gene of the epidermal growth factor gene family, leads to in vitro transformation of a normal mouse mammary epithelial cell line. Cancer Res. *51,* 1051-1054.
8. Ciardiello, F., Tortora, G., Bianco, C., Selvam, M.P., Basolo, F., Fontanini, G., Pacifico, F., Normanno, N., Brandt, R., Persico, M.G., Salomon, D.S., and Bianco, A.R. (1994). Inhibition of CRIPTO expression and tumorigenicity in human colon cancer cells by antisense RNA and oligodeoxynucleotides. Oncogene *9,* 291-298.
9. Shen, M.M., Wang, H., and Leder, P. (1997). A differential display strategy identifies *Cryptic,* a novel EGF-related gene expressed in the axial and lateral mesoderm during mouse gastrulation. Development *124,* 429-442.
10. Kinoshita, N., Minshull, J., and Kirschner, M.W. (1995). The identification of two novel ligands of the FGF receptor by a yeast screening method and their activity in Xenopus development. Cell *83,* 621-630.
11. Lohmeyer, M., Harrison, P.M., Kannan, S., DeSantis, M., O'Reilly, N.J., Sternberg, M.J.E., Salomon, D.S., and Gullick, W.J. (1997). Chemical synthesis, structural modeling, and biological activity of the epidermal growth factor-like domain of human *Cripto.* Biochemistry *36,* 3837-3845.
12. Brandt, R., Normanno, N., Gullick, W.J., Lin, J.H., Harkins, R., Schneider, D., Jones, B.W., Ciardiello, F., Persico, M.G., Armenante, F., Kim, N., and Salomon, D.S. (1994).

Identification and biological characterization of an epidermal growth factor-related protein: *cripto-1*. J. Biol. Chem. *269*, 17320-17328.

13. Kannan, S., De Santis, M., Lohmeyer, M., Riese II, D.J., Smith, G.H., Hynes, N., Seno, M., Brandt, R., Bianco, C., Persico, G., Kenney, N., Normanno, N., Martinez-Lacaci, I., Ciardello, F., Stern, D.F., Gullick, W.J., and Salomon, D.S. (1997). Cripto enhances the tyrosine phosphorylation of Shc and activates mitogen-activated protein kinase (MAPK) in mammary epithelial cells. J. Biol. Chem. *272*, 3330-3335.

14. Kuniyasu, H., Yoshida, K., Yokozaki, H., Yasui, W., Ito, H., Toge, T., Ciardiello, F., Persico, M.G., Saeki, T., and Salomon, D.S. (1991). Expression of cripto, a novel gene of the epidermal growth factor family, in human gastrointestinal carcinomas. Japan. J. Cancer Res. *82*, 969-973.

15. Qi, C.F., Liscia, D.S., Normanno, N., Merlino, G., Johnson, G.R., Gullick, W.J., Ciardiello, F., Saeki, T., Brandt, R., Kim, N., Kenney, N., and Salomon, D.S. (1994). Expression of transforming growth factor alpha, amphiregulin and cripto-1 in human breast carcinomas. Br. J. Cancer *69*, 903-910.

16. Groenen, L.C., Nice, E.C., and Burgess, A.W. (1994). Structure-function relationships for the EGF/TGF-alpha family of mitogens. Growth Factors *11*, 235-257.

17. Amaya, E., Musci, T.J., and Kirschner, M.W. (1991). Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in *Xenopus* embryos. Cell *66*, 257-270.

18. Amaya, E., Stein, P.A., Musci, T.J., and Kirschner, M.W. (1993). FGF signalling in the early specification of mesoderm in *Xenopus*. Development *118*, 477-487.

19. Daub, H., Weiss, F.U., Wallasch, C., and Ullrich, A. (1996). Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. Nature *379*, 557-560.

20. McFarlane, S., Cornel, E., Amaya, E., and Holt, C.E. (1996). Inhibition of FGF receptor activity in retinal ganglion cell axons causes errors in target recognition. Neuron *17*, 245-254.

21. Saffell, J.L., Williams, E.J., Mason, I.J., Walsh, F.S., and Doherty, P. (1997). Expression of a dominant negative FGF receptor inhibits axonal growth and FGF receptor phosphorylation stimulated by CAMS. Neuron *18*, 231-242.

22. Hogan, B., Beddington, R., Costantini, F., and Lacy, E. (1994). *Manipulating the mouse embryo*. 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

23. Shen, M.M., Skoda, R.C., Cardiff, R.D., Campos-Torres, J., Leder, P., and Ornitz, D.M. (1994). Expression of LIF in transgenic mice results in altered thymic epithelium and apparent interconversion of thymic and lymph node morphologies. EMBO J. *13*, 1375-1385.

24. Taketo, M., Schroeder, A.C., Mobraaten, L.E., Gunning, K.B., Hanten, G., Fox, R.R., Roderick, T.H., Stewart, C.L., Lilly, F., Hansen, C.T., and Overbeek, P.A. (1991). FVB/N: an inbred mouse strain preferable for transgenic analyses. Proc. Natl. Acad. Sci. USA *88*, 2065-2069.

25. Muller, W.J., Lee, F.S., Dickson, C., Peters, G., Pattengale, P., and Leder, P. (1990). The int-2 gene product acts as an epithelial growth factor in transgenic mice. EMBO J. *9*, 907-913.

26. Jhappan, C., Gallahan, D., Stahle, C., Chu, E., Smith, G.H., Merlino, G., and Callahan, R. (1992). Expression of an activated Notch-related int-3 transgene interferes with cell

differentiation and induces neoplastic transformation in mammary and salivary glands. Genes Dev. *6,* 345-355.

27. Kinsella, T.M., and Nolan, G.P. (1996). Episomal vectors rapidly and stably produce high-titer recombinant retrovirus. Hum. Gene Ther. *7,* 1405-1413.

28. Cepko, C., and Pear, W. (1996). "Transduction of genes using retroviral vectors", in *Current Protocols in Molecular Biology,* F.M. Ausubel, *et al.,* eds. John Wiley & Sons: New York. pp. 9.91-9.14.6.

29. Cheng, H.-J., and Flanagan, J.G. (1994). Identification and cloning of ELF-1, a developmentally expressed ligand for the Mek4 and Sek receptor tyrosine kinases. Cell *79,* 157-168.

30. Flanagan, J.G., and Leder, P. (1990). The kit ligand: a cell surface molecule altered in steel mutant fibroblasts. Cell *63,* 185-194.

31. Cheng, H.-J., Nakamoto, M., Bergemann, A.D., and Flanagan, J.G. (1995). Complementary gradients in expression and binding of ELF-1 and Mek4 in development of the topographic retinotectal projection map. Cell *82,* 371-381.

32. Dono, R., Scalera, L., Pacifico, F., Acampora, D., Persico, M.G., and Simeone, A. (1993). The murine cripto gene: expression during mesoderm induction and early heart morphogenesis. Development *118,* 1157-1168.

33. Wang, T.C., Cardiff, R.D., Zukerberg, L., Lees, E., Arnold, A., and Schmidt, E.V. (1994). Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. Nature *369,* 669-671.

34. Pear, W.S., Nolan, G.P., Scott, M.L., and Baltimore, D. (1993). Production of high-titer helper-free retroviruses by transient transfection. Proc. Natl. Acad. Sci. USA *90,* 8392-8396.

35. Tartaglia, L.A., Dembski, M., Weng, X., Deng, N., Culpepper, J., Devos, R., Richards, G.J., Campfield, L.A., Clark, F.T., Deeds, J., Muir, C., Sanker, S., Moriarty, A., Moore, K.J., Smutko, J.S., Mays, G.G., Woolf, E.A., Monroe, C.A., and Tepper, R.I. (1995). Identification and expression cloning of a leptin receptor, OB-R. Cell *83,* 1263-1271.

36. Müller, U., Wang, D., Denda, S., Meneses, J.J., Pedersen, R.A., and Reichardt, L.F. (1997). Integrin $\alpha 8\beta 1$ is critically important for epithelial-mesenchymal interactions during kidney morphogenesis. Cell *88,* 603-613.

37. Johnson, S.E., Rothstein, J.L., and Knowles, B.B. (1994). Expression of epidermal growth factor family gene members in early mouse development. Dev. Dyn. *201,* 216-226.