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CRIPTO: A TARGET for BREAST CANCER TREATMENT**INTRODUCTION**

This study was delayed in starting by one year because of lack of a suitable post-doctoral associate to perform the laboratory work. Dr Min Li has now been working on the project since July, 2002, and we are reporting the results of the first year's work.

The literature meanwhile has described a few important advances of how Cripto interacts with the mammary cell. Cripto (CR1 or Cr1 (mouse)) acts as both a ligand and a co-receptor in the stimulation of cells and the generation of a signal. As a ligand it binds to the Activin receptor (Act1B) also called ALK4, in a complex with *Nodal* a TGF β 1 related gene and ACT11B, to activate signals through SMAD2 transcription factor which then regulates cell responses through its target genes. This pathway exists in the embryo as well as in the mammary epithelium (Bianco et al 02). In normal developing mammary cells, this is the predominant and possibly the only pathway. In addition, CR1 can also activate a distinct pathway, by activating AKT and MAPK independently of *Nodal*. A new hypothesis that we suggest is that in breast cancer cells, the CR1/AKT/MAPK/ ERK pathway may also play a major role in the transformed character of the cells that leads to excess proliferation.

BODY OF REPORT

Cripto is a growth factor that is important in breast development during puberty, during pregnancy and lactation, as well as breast cancer, leading to increases in cell proliferation and to increased survival of cells. These results were obtained for human mammary cells by several groups including ours using mouse CID9 mammary epithelial cells derived from a mid-term pregnant mouse [1]. It consists of three major domains, an EGF-like, a CFC domain and a Carboxy-terminal lipid linkage motif using the lipid GPI to link CR1 to the membrane. It is known that the EGF domain is required for the stimulation of the growth of mammary cells [2, 3]. We have chosen the CFC domain to make mutant CR1 because it has not been explored well yet.

For the current studies we decided to use the NMuMG mammary line because these cells do not express Cripto, being derived from an adult mouse mammary gland. But they do express receptors for Cripto. The idea is that expression of Cripto and mutant forms of Cripto will increase proliferation of the cells and a mutant that has a reduced activity will not or may even inhibit growth if it acts as a dominant negative. Therefore there are several ways that a mutant may be designed. We have chosen to test the putative three main cysteine loops in the CFC domain to mutate, first mutating the DNA sequence comprising each loop in two types of vector:-

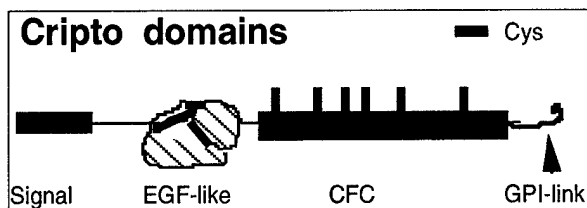
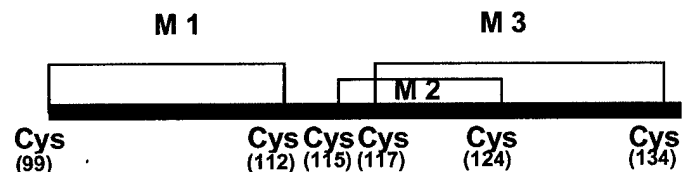
Constructs made

1. From a collaborator we obtained pcDNA3-Cr1, =Full-length Cripto expression vector driven by the CMV promoter.
2. From another source we got pcDNA3-HA-Cr1 = NT-inserted HA-tag on full-length Cr1 and pcDNA3-FLAG-Cr1 =NT inserted FLAG tag on full-length Cripto

Dr Min Li made these mutants in 6 locations in the CFC domain (red)-

3. M1 mutant, pcDNA3-Cr1(C99A/C112A) = mutated first Cys-Cys loop (C1 and C2)
4. M2 mutant, pcDNA3-Cr1(C115A/C124A) =mutated second Cys-Cys loop (C3 and C5)
5. M3 mutant pcDNA-Cr1 (C117/C133A) =mutated third Cys-Cys loop (C4 and C6)

Figure 1. The domains in Cripto

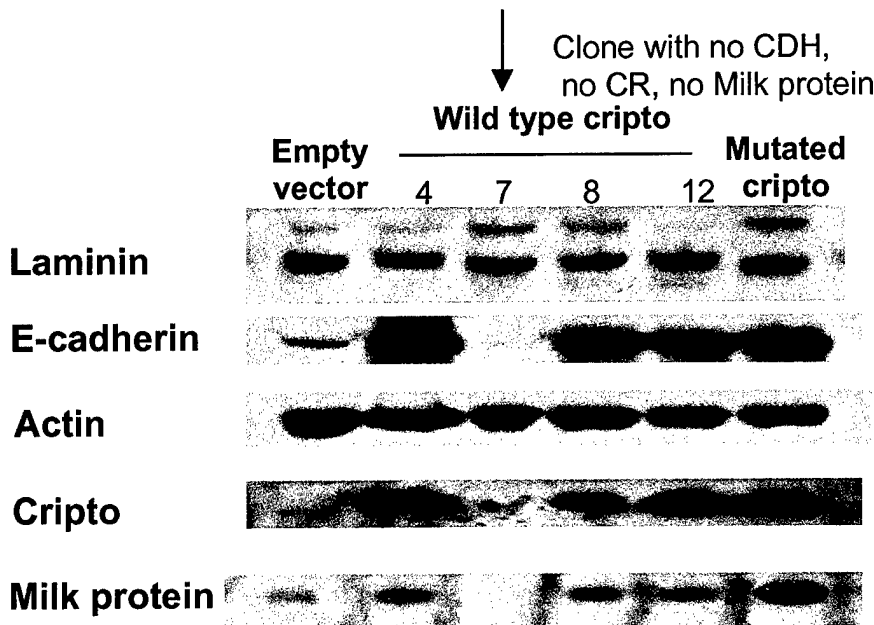
**Possible Structure of the CFC domain**

For each mutated Cripto,
2 Cysteines were replaced by Ala

Expt 1. The Expression vectors were transfected into NMuMG normal adult mouse mammary epithelial cells and stable neo-resistant clones were produced and grown up. Western blots showed that they expressed Cr1, a protein of 27 kD.

The result was encouraging at first because the cells had differing appearances, some looked transformed and some looked epithelial. We concluded that there may be epithelial-mesodermal conversion. We tested the expression of various markers such as E-cadherin, Keratin 8 and 18, collagen I and laminin, Figure 2

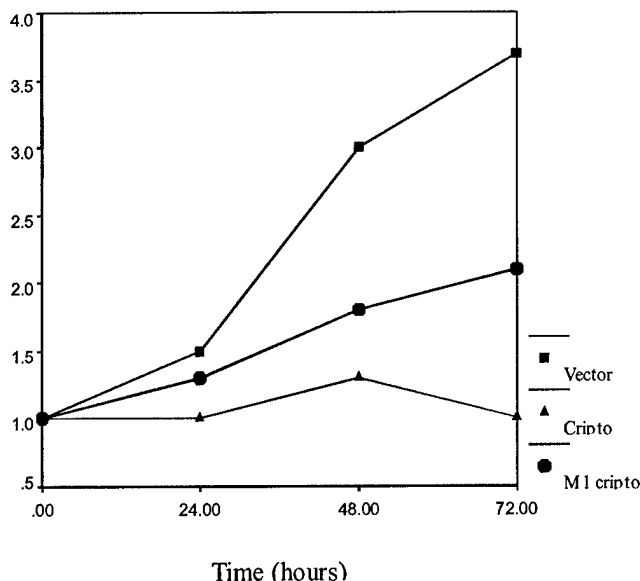
The phenotype of NMuMG cell stably expressing vector, Cr-1 and M1-Cr-1



and the result (Fig 2) was not helpful. Upon making a literature search, we found the answer. A published paper indicated that the NMuMG cell line is heterogeneous and consists of a mixture of two cell types., only one is epithelial (cadherin +) and the other is mesenchymal. Therefore our analyses could not be interpreted with confidence. Clone 7 was obviously a mesenchymal cell type

Figure 3 (Y axis represents the number of cells)

Growth curve of NMuMG, NMuMG Cr-1 and NMuMG M1-Cr-1 cells in media

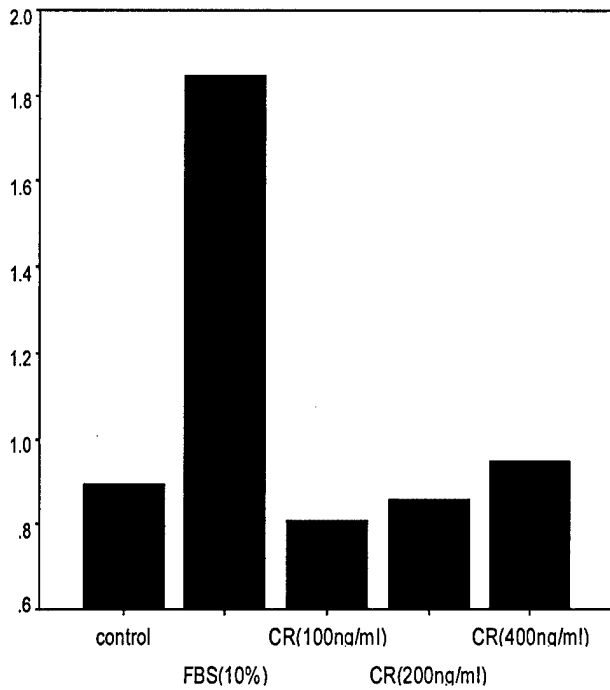


Expt 2. The effect of mutant Cr expression on cell proliferation. Cripto has been reported to stimulate proliferation of mammary cells of several species. We would expect normal Cr1 to stimulate and the mutants to have no effect or to inhibit growth. The results did not indicate a clear effect on cell growth of NMuMG clones. Fig 3 shows that in low serum, Cripto expression reduced the proliferation rate of the NMuMG clone 12 cells. The mutant expressing M1 mutated cripto grew better but not as well as vector control cells. The explanation cannot be explained on the clone being mesenchymal in type because cl12 is epithelial.

Moreover, the addition of Soluble Cripto to the medium of NMuMG cells also had no effect on the survival of the cells cultured in serum-free medium (data not shown). This result was unexpected since we expected NMUMG to respond to Cr1 by increased growth rates or increased survival.

Figure 4 (Y axis represents the number of cells)

The effect of recombinant cripto on the growth of MCF7 cells



The result was that recombinant Cripto had little effect on MCF7 grown in serum-free medium, while serum addition caused a large improvement in growth rate (Fig 4). Thus the cells are responsive to other growth stimuli

Figure 5 (below)

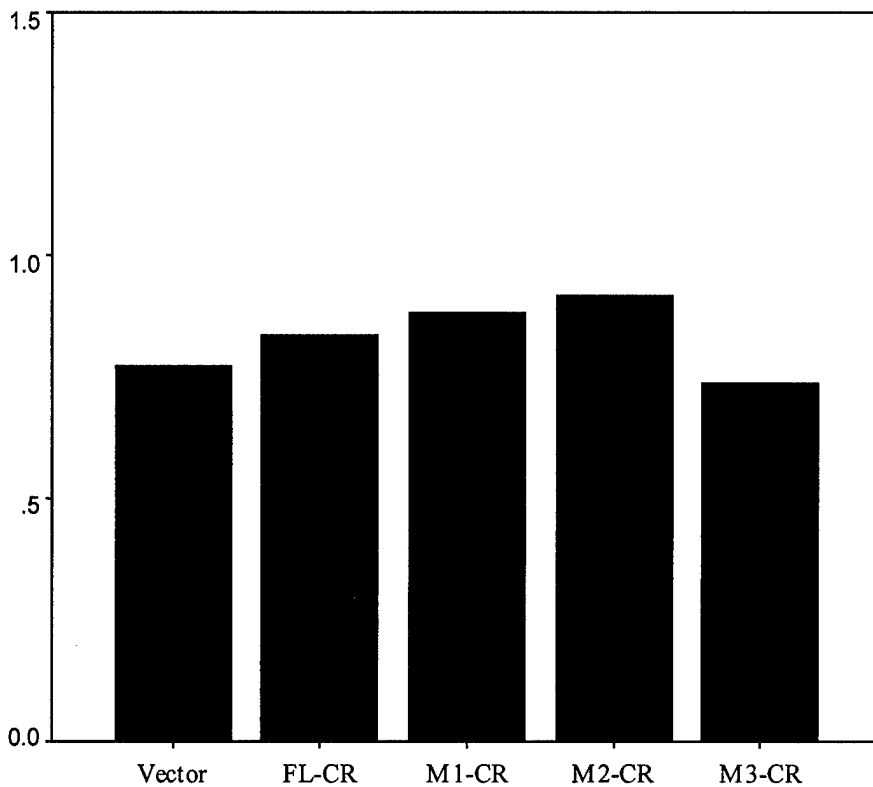


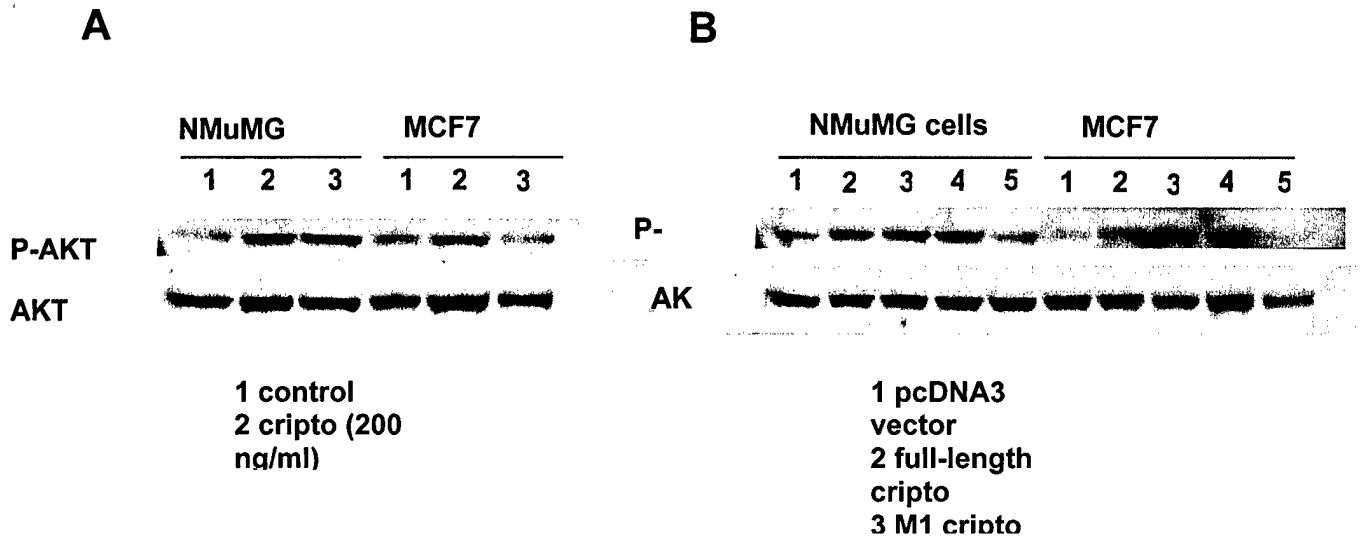
Figure 5 The effect of Transient-expressing Cripto on the growth of NMuMG cells after 24h In 10% serum then 48h in serum-free medium

Expt 3. The effect of transiently expressed Cripto and mutant Cr1 was then measured in MCF7 cells to determine if the recombinant Cr1 was inactive. There was little activity on MCF7 cells thus indicating that these cells are refractive to Cr1 as a growth factor. However, there was a small growth inhibitory effect of Mutant 3 on NMuMG cells (Fig 5), that may not be real and needs to be evaluated further.

Expt 4. We tested if phospho Akt was activated by Cripto expressed constitutively in NMuMG clones. Can Cr1 stimulate the activation of AKT phosphorylation of the signaling intermediate, p-Akt (Fig 6)?

Figure 6

Cripto induced AKT phosphorylation
 (A measured in serum-free cells for 6h then Cripto for 30 min)
 (B measured 48h after transfection, then serum-free for 30min)



A Cripto directly added into the media of NMuMG and MCF7 cells
 B Transient expression of cripto vector in NMuMG and MCF7 cells

B shows the effect of the transfection of Cr1 vectors on signaling by P-AKT. Note that mutant 3 (lane 5) does not signal to p-AKT in both NMuMG and in MCF7 cells.

This result suggests how to investigate the effect of mutant forms of Cripto in the coming year.

Accomplishments

- Determined that NMuMG is unsuitable as a mammary cell model because of the heterogeneity of cell types in the culture. We will use Eph4 cloned mammary epithelial cells instead
- Shown that Cripto signals through Phospho-Akt in both NMuMG and MCF7 cells.

Reportable Outcomes

- Following the SOW, we have obtained 2 constructs from other workers and made three mutated forms of Cripto in a CMV directed expression vector (SOW #1).
- Transfected the constructs into a mouse cell line and prepared neo-resistant cell lines and cloned them (SOW #2). We have also transfected a human cell line for comparisons.
- We have measured the proliferation rate, the apoptosis rate and the survival rate of each cell type and used Western blotting to measure a selection of proteins that should be expressed (SOW #3)

CONCLUSIONS

We have found that a mutant (M3) with an altered third Cystine bridge cannot signal appropriately in either of these cell lines (SOW#4). This mutant will be examined in more detail in the coming year.

We have obtained another cell line that was cloned from NMuMG, called EpH4. We will do all further studies in this cloned cell line in which we should be able to obtain consistent results. This will lead to an improvement in the data results which were diluted by the heterogeneity of the NMuMG cell line.

Continuing Studies in Year 2.

Since we have narrowed down the portion of the CFC domain of Cripto that is sensitive to mutation, we will continue with the synthesis of peptides to test their ability to block normal Cripto activation. This is the exciting but risky part of the project. Will a peptide, open or constrained have an effect on normal Cripto activity in mammary cells.

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