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13. ABSTRACT *(Maximum 200 words)*

Cripto is a member of the EGF-CFC gene family and is involved in mammary tumorigenesis. My research project focus on identifying and cloning of CRIPTO receptors. In order to detect CRIPTO specific binding protein, a co-immunoprecipitation assay using AP-mCRIPTO as affinity reagent has been established. Using this assay, two protein bands, a major 65 Kd band and a weaker 63 Kd band, were detected as specifically binding to AP-mCRIPTO but not AP. These two proteins were also shown to be cell surface proteins, suggesting that they could be CRIPTO receptors. The purification and sequencing of these two proteins are underway.

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5. Introduction

5.1. Background and Significance

Cripto is a member of the EGF-CFC gene family. It shares an EGF-like consensus sequence containing six cysteine residues in a region of approximately 40 amino acids with all EGF family members. However, the EGF-like motif in CRIPTO is significantly different from the classical EGF motif in that all seven residues between the first and second cysteines and five residues between the third and fourth cysteines are absent [1], indicating that the CRIPTO EGF motif may have a distinct structure. Consistent with this feature, experiments have shown that CRIPTO does not interact with all of the four known members of the *erb*B receptor family -- EGF receptor, *erb*B-2, *erb*B-3 and *erb*B-4, suggesting that CRIPTO binds to an as yet unidentified receptor [2]

CRIPTO has a growth factor activity and is involved in mammary tumorigenesis. Surveys of a large number of human primary ductal and lobular breast carcinoma specimens have demonstrated that *Cripto* is overexpressed in approximately 80% of the tumors examined, but not in adjacent noninvolved mammary tissues [3, 4]. Overexpression of *Cripto* was also found in mouse mammary tumors [5, 6]. Human CRIPTO protein secreted by transfected CHO cells or the 47-mer refolded peptide containing the CRIPTO EGF-like motif can stimulate the proliferation of several human breast cancer cell lines, including MDA-MB-453 and SK-BR-3, as well as the untransformed mammary epithelial cell line 184A1N4 [7]. Transfection of human *Cripto* results in transformation of and NOG-8 mouse normal mammary epithelial cells [8]. Experiments using the refolded CRIPTO peptide have shown that CRIPTO can induce tyrosine phosphrylation of the SH2 adapter protein Shc in HC-11 cells, resulting in increased association of Shc with Grb2 and elevated MAPK kinase activity [2, 9]. More recently it has been shown that recombinant CRIPTO can transactivate *erb*B-4 receptor, but not EGF receptor, *erb*B-2 and *erb*B-3 [10]. These results suggest that *Cripto* may be involved in a *Ras* signaling pathway.

5.2. Scope and purpose of research

Although *Cripto* is frequently overexpressed in breast cancers, the direct relationship between *Cripto* overexpression and mammary tumorigenesis is still unclear. Because the identity of the *Cripto* receptor is still unknown, the mechanism and consequences of *Cripto* signaling are poorly understood. My research focuses on identifying and cloning *Cripto* receptor to investigate its putative oncogenic activity. In our laboratory, we have established a receptor binding and co-immunoprecipitation assay using a CRIPTO fusion protein for this purpose. The cloning of a *Cripto* receptor will allow further study of *Cripto* signal transduction pathway.

6. Body

6.1. Materials and methods

6.1.1. Binding assay

In order to study the interaction between *Cripto* and its receptor, we have generated a soluble receptor affinity reagent that consists of CRIPTO protein fused to secreted human placental alkaline phosphatase (AP). The advantage of using a AP fusion protein as a

receptor affinity reagent is that it is nonradioactive, sensitive and the AP fusion protein can be easily detected and quantitated. Human placental AP is heat stable. Therefore, the endogenous AP activity can be inactivated by heating. AP-hCRIPTO supernatant and AP supernatant were prepared by transiently transfecting the corresponding constructs or vector into COS7 cells using lipofectamine. Cell culture supernatants were collected after 72 hrs. AP activity was determined in a microplate colorimeter assay using p-nitrophenyl phosphate as substrate. 1 unit AP activity was defined as the ability to result in 1 OD_{405} per hour at maximum rate under standard conditions [11].

Because the MAPK activity in mouse mammary epithelial HC-11 cells can be activated by human CRIPTO and differentiation of HC-11 cells can be inhibited by CRIPTO, I chose HC-11 cells to perform quantitative cell binding assays for receptor detection. $3.5x10^5$ HC-11 cells were suspended in 1 ml AP-CRIPTO supernatant or AP supernatant (2000 U/ml), as a control, and incubated at room temperature on a rotator for 90 minutes. After washing, cells were lysed and nuclei were spun down. Then the supernatants were heated at 65°C for 10 minutes to inactivate endogenous AP activity. The AP activity in the lysate supernatant was assayed by the standard method [11]

Several EGF related member growth factors can bind heparin. In order to determine if CRIPTO is a heparin binding protein, I performed heparin-bead binding assay using AP-CRIPTO. 0.15 ml heparin-beads were mixed with 1 ml AP-CRIPTO supernatant or AP supernatant (1000 U/ml), as a control, and incubated at room temperature, on a rotator for 60 minutes. After four times washing with 1xPBS, the bound AP-CRIPTO or AP were eluted with 0.3 ml 1xPBS/0.35M NaCl. The AP activity in the eluates was assayed by the standard method [11].

6.1.2. Co-immunoprecipitation Assay

Using AP-mCRIPTO supernatant, I also performed co-immunoprecipitation assay to detect the CRIPTO binding proteins. Again, HC-11 cells were used as target cells. I grew the HC-11 cells to about 80% confluence in 100mm dishes and labeled the cells using ^{35}S-methionine. The HC-11 cells in each dish were lysed with 3 ml 1xRipa buffer. 0.5 ml radio-labeled HC-11 lysate were mixed with 0.3 ml AP-mCRIPTO supernatant or AP supernatant (2500 U/ml), as a control, and incubated at 4°C for 60 minutes. Then 5 µl AP monoclonal antibody (sigma) was added to the mixture and continued to incubated for another 60 minutes. Finally 30 µl formalin-fixed Staph A cells (Calbiochem) were added to the mixture for immunoprecipitation by incubating at 4°C for 20 minutes. The Staph A cell pellets were washed 4 times with 1xRipa buffer and treated with 30 µl 1x SDS sample buffer. The immunoprecipitates were then loaded on a 10% SDS-PAGE gel for analysis.

In order to detect any cell surface CRIPTO binding protein, I labeled HC-11 cells with 0.25 mg/ml sulfo-NHS-LC-biotin (Pierce) which only biotinylates cell surface proteins, followed by preparation of cell lysate [12]. Using the biotinylated lysate, the same co-immunoprecipitation assay as described for radio-labeled lysate was performed. The immunoprecipitates were resolved by 10% SDS-PAGE, transferred onto PVDF membrane, and detected with avidin-HRP conjugate.

6.2. Results and discussion

6.2.1. Binding assay

Previously, specific human CRIPTO binding sites have been detected on HC-11, MDA-MB-453 and SK-BR-3 cells [2]. In our laboratory we also demonstrated the binding of AP fused human CRIPTO to these cells. Scatchard analysis of quantitative binding assay indicated a Kd of about 10 nM for binding interaction and about $2.5x10^5$ binding sites per cell.

Because we work on mouse models, I performed this binding assay using AP fused mouse CRIPTO. We surprisingly found that the AP-mCRIPTO did not bind to HC-11 cells (Figure 1A). The level of AP-mCRIPTO binding to HC-11 cells was at least 20-fold lower than that of AP-hCRIPTO. Because some growth factors in EGF family can bind heparin, one possibility was that human CRIPTO can bind to heparan sulfate, a common component of extracellular matrix, while mouse CRIPTO does not. The results from a heparin-bead binding assay showed that this was the case (Figure 1B). The AP-hCRIPTO strongly binds to heparin-beads, but the binding of AP-mCRIPTO was similar to the AP control. Therefore human CRIPTO and mouse CRIPTO differ in their ability to bind heparin. Our binding data suggests that the binding sites on HC-11 cells for human CRIPTO could correspond to heparan sulfate proteoglycans.

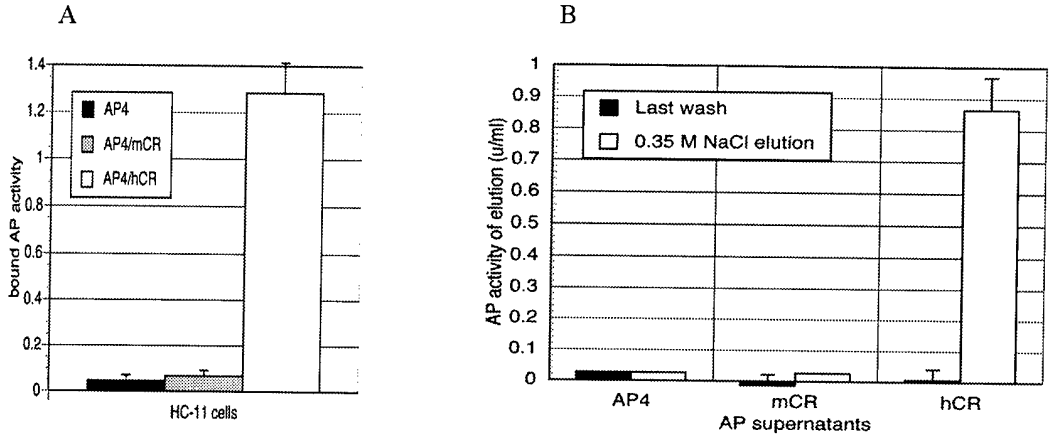

Figure 1. Quantitative binding assay. **(A)** Binding of mouse CRIPTO and human CRIPTIO to HC-11 Cells. $3.5x10^5$ HC-11 cells were suspended in 1 ml AP-mCRIPTO or AP-hCRIPTO supernatant (2000 U/ml). After incubation and wash, the cells were lysed. The cell lysates were heated at 65°C for 10 minute to inactivate endogenous AP activity and then the bound AP activity was detected as described [11]. AP supernatant (2000 U/ml) was used as a control. **(B)** Binding of mouse CRIPTO and human CRIPTIO to heparin-beads. 0.15 ml heparin-beads were mixed with 1 ml AP-mCRIPTO or AP-hCRIPTO supernatant (1000 U/ml) and incubated at room temperature for 60 minutes. After washing with 1xPBS, the bound AP-CRIPTO was eluted with 0.3 ml 1xPBS/0.35M NaCl. The AP activity in the elutes was assayed [11]. AP supernatant (1000 U/ml) was used as a control.

6.2.2. Co-immunoprecipitation assay

We are interested in the function of mouse CRIPTO and the identification of its receptor. Because CRIPTO has biological activity on mouse mammary HC-11 cells [2, and our unpublished data], we believe CRIPTO receptors should exist on HC-11 cells. The failure to detect binding of AP-mCRIPTO to HC-11 cell may be due to the low

sensitivity of the cell binding assay. Therefore I decided to use AP-mCRIPTO to perform a sensitive co-immunoprecipitation assay to identify mouse CRIPTO specific binding proteins.

In the co-immunoprecipitation assay using ^{35}S-methionine labeled HC-11 cell lysate, two protein bands, a major 65 Kd band and a weaker 63 Kd band, were detected as specifically binding to AP-mCRIPTO but not AP (Figure 2A). This indicated the two proteins are mouse CRIPTO specific binding proteins. In order to know if the two mouse CRIPTO binding proteins are cell surface proteins, I also performed co-immunoprecipitation assay using biotin-labeled HC-11 lysate in which only cell surface

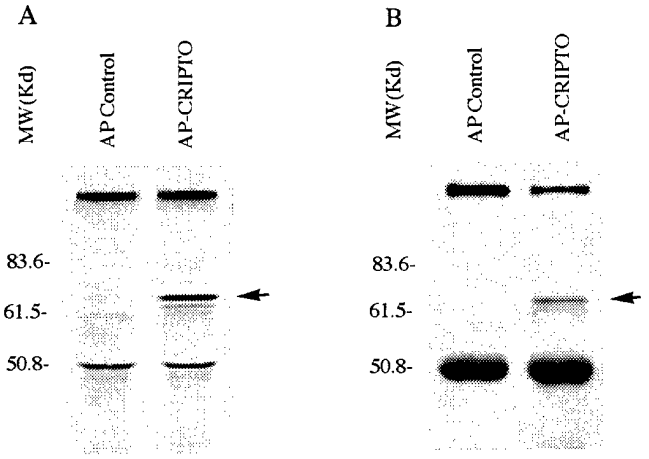

Figure 2. Co-immunoprecipitation assay. (A) Co-immunoprecipitation assay using radio-labeled HC-11 cell lysate. 0.5 ml radio-labeled HC-11 lysate were mixed with 0.3 ml AP-mCRIPTO supernatant or AP supernatant (2500 U/ml) as a control. After incubation at 4°C for 60 minutes, AP-mCRIPTO or AP were immunoprecipitated with 5 µl AP monoclonal antibody and 30 µl Staph A cells. The precipitates were resolved by 10% SDS-PAGE. **(B)** Co-immunoprecipitation assay using biotin-labeled HC-11 cell lysate. HC-11 cells were labeled with 0.25 mg/ml sulfo-NHS-LC-biotin which only biotinylates cell surface proteins and then were lysed with 1xRipa buffer. The immunoprecipitation assay was exactly done as for radio-labeled HC-11 cells. The immunoprecipitates were resolved by 10% SDS-PAGE, transferred onto PVDF membrane, and detected with avidin-HRP conjugate [12].

proteins were labeled. In this assay, two proteins with the same molecular weight as the two detected in radio-labeled co-immunoprecipitation assay have been identified (Figure 2B). This result suggested that the 65Kd, and 63Kd mouse CRIPTO binding proteins are surface proteins and could be CRIPTO receptors. I am now going to purify and sequence the two proteins to determine their identity.

7. Conclusion

The major progress I made in the past year is the identification of the 65Kd and 63Kd CRIPTO specific binding proteins. Because they are cell surface proteins, they may represent CRIPTO receptors. The purification and sequencing of these two proteins are underway.

8. Reference

1. 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.
2. 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.
3. 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.
4. Panico, L., D'Antonio, A., Salvatore, G., Mezza, E., Tortora, G., De, L.M., De, P.S., Giordano, T., Merino, M., Salomon, D.S., Mullick, W.J., Pettinato, G., Schnitt, S.J., Bianco, A.R., and Ciardiello, F. (1996). Differential immunohistochemical detection of transforming growth factor alpha, amphiregulin and CRIPTO in human normal and malignant breast tissues. Int. J. Cancer *65,* 51-56.
5. Kenney, N.J., Huang, R.P., Johnson, G.R., Wu, J.X., Okamura , D., Matheny, W., Kordon, E., Gullick, W.J., Plowamn, G., Smith, G.H., Salomon, D.S., and Adamson, E.D. (1995). Detection and Location of Amphiregulin and Cripto-1 Expression in the Developing Postnatal Mouse Mammary Gland. Mol. Reproduct. Develop. *41,* 277-286.
6. Herrington, E.E., Ram, T.G., Salomon, D.S., Johnson, G.R., Gullick, W.J., Kenney, N., and Hosick, H.L. (1997). Expression of Epidermal Growth Factor-Related Preteins in the Aged Adult Mouse Mammary Gland and Their Relationship to Tumorigenesisi. J. cellular Physiology *170,* 47-56.
7. 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.
8. 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.
9. 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.
10. Bianco, C., Kannan, S., Santis, M.D., Seno, M., Tang, C.K., Martinez-Lacaci, I., Kim, N.,Wallace-Jones, B., Lippman, M.E., Eber, A.D., Wechselberger, C., and Salomon, D. (1999). Cripto-1 indirectly stimulates the tyrosine phosphorylation of *erb*B-4 through a noval receptor. J. Biol. Chem. *274*, 8624-8629

11. Berger, J., Hauber, J., Hauber, R., Geiger, R., and Cullen, B.R. (1988). Secreted placental alkaline phosphatase: a powerful new quantitative indicator of gene expression in eukaryotic cells. Gene *66,* 1-10.
12. Altin, J.G., and Pagla,E.B. (1995). A one-step procedure for biotinylation and chemical cross-linking of lymphocyte surface and intracellular membrane-associated molecules. Anal. Biochem. *244*, 382-289.