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Cooperation of $\alpha 6\beta 4$ and $\alpha 3\beta 1$ Integrin Receptors in Breast
Carcinoma Invasion

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Introduction

Our research focuses on one of the key issues in breast carcinoma biology, the interaction of the breast carcinoma cell with its surroundings. Carcinoma cells actively produce extracellular matrix components, especially laminins, and use their integrin receptors for adhering to these newly deposited matrix proteins (Tani et al., 1999; Tani et al., 1997). Adhesion to laminins and other matrix molecules not only provides the cells with support and physically enables cell migration, but also provides signals that regulate carcinoma cell growth, differentiation, invasion through surrounding tissues and eventually metastasis. Our main focus, the integrin receptors, is important for breast carcinoma progression in at least two ways. Upon ligation by extracellular matrix molecules integrins activate specific signal transduction pathways, including PI3-kinase, p42/p44 MAP kinase and p38 MAP kinase (Ivaska et al., 1999; Shaw et al., 1997; Wei et al., 1998). On the other hand, integrins modulate the immediate vicinity of the cells, e.g. by concentrating laminin heterotrimers near the plasmamembrane thus facilitating laminin polymerisation and basement membrane formation.

During the two years with Taneli T. Tani as the PI of this project, two major projects on laminin receptor function were completed. The first project aimed at finding cytoplasmic proteins specifically binding to the type I PDZ recognition sequence at the C-terminus of $\alpha 6A$ integrin subunit. Surprisingly, TIP-2/GIPC was found to interact with both $\alpha 6$ isoforms, and the $\alpha 6B$ subunit was demonstrated to have a novel type of PDZ recognition sequence. The second project was carried out in collaboration with Dr. Leslie M. Shaw. Dr. Shaw had preliminary data on the role of PKC ϵ in carcinoma cell motility and invasion. On the other hand, my own preliminary data pointed towards synergistic effects of RhoA and PKC activation in invading cells. Taken together, our data shows that PKC ϵ provides a parallel signal regulated in part by PI3K that facilitates the RhoA driven migration and invasion of carcinoma cells.

Body

Because our previous data suggested that $\alpha 6A$ and $\alpha 6B$ cytoplasmic domains are likely to have specific signaling functions, we took this as our starting point in characterizing the interplay on laminin receptor function in breast carcinoma. As is true for other integrin α subunits, the cytoplasmic domains of $\alpha 6A$ and $\alpha 6B$ are short (36 and 56 amino acids, respectively), devoid of enzymatic activities and docking sites for cell signaling molecules. Surprisingly little is known about direct protein-protein interactions between integrin α -subunits and cytoplasmic proteins. However, we realized that there is a classical type I PDZ domain at the end of $\alpha 6A$ integrin subunit (TSDA*), characterized by serine at -2 position and an aliphatic residue at the C-terminal 0 position. Yeast two-hybrid screen suggested that TIP-2/GIPC, a previously characterized cytoplasmic protein with a single PDZ domain, binds to the C-terminal sequences of $\alpha 6A$, $\alpha 5$ (TSDA*) and $\alpha 6B$ (ESYS*) integrins. The recognition sequence in $\alpha 6B$ (ESYS*) has not been previously characterized as a PDZ recognition sequence, and probably represents a new subclass of type I PDZ binding site.

This project on the putative PDZ domains in integrin α subunits was finished during the period of May 15, 2001 to May 14, 2002. In addition to the data provided in the first annual report, a definitive proof of the PDZ interaction was provided by demonstrating that the PDZ domain of TIP-2/GIPC is sufficient for interaction with both $\alpha 6A$ and $\alpha 6B$ integrin subunits. In addition, the properties of the novel PDZ recognition sequence in $\alpha 6B$ C-terminus were characterized by a more thorough point mutation analysis. The results were published in September 2001 (Tani and Mercurio, 2001).

Most of the work during the period of May 15, 2001 to May 14, 2002 focused on the role PKCs in breast carcinoma cell invasion. Previous studies have shown that $\alpha 6\beta 4$ integrin activates PI3-K, and eventually leads to facilitated carcinoma cell migration and invasion (Shaw et al., 1997). Several lines of evidence suggest a role for PKCs in the regulation of cell migration, and because novel and atypical PKCs are regulated by PI3-K (Cenni et al., 2002; Chou et al., 1998; Derman et al., 1997) we hypothesized that one or more of these kinases might act downstream of the $\alpha 6\beta 4$ /PI3-K pathway. Some of our own unpublished data also supported this hypothesis. Clone A colon carcinoma cells do not form lamellae on collagen I. However, a short treatment with PMA rapidly induces large lamellae and rapid cell migration even on collagen I. This phenomenon is accompanied by activation of RhoA. On the other hand, we had preliminary data that kinase inactive alleles of PKC ϵ inhibit haptotactic migration and invasion of MDA-MB-435 breast carcinoma cells, whereas kinase inactive isoforms of other PKCs fail to do this.

The preliminary biochemical experiments on RhoA and PKCs were done with Clone A cells, and therefore we started the study by characterizing the expression pattern of different PKC isoforms in Clone A cells. Novel PKCs δ and ϵ are expressed in Clone A cells, whereas η and θ are not. The atypical PKC ζ , also regulated by PI3-K, is expressed in Clone A cells.

Although Clone A cells do not invade in Boyden chamber models of invasion, they do show haptotactic migration towards a laminin-1 gradient. We expressed several PKC constructs in Clone A cells and compared their ability for haptotactic migration. Kinase inactive PKC ϵ reduced haptotactic migration by 80%, whereas the kinase inactive PKC δ and PKC ζ constructs had no effect. This result suggests that PKC ϵ has a unique role among PKCs in facilitating the migration of carcinoma cells.

As the next step, we aimed at characterizing the activation of PKC ϵ in different conditions. Activation of PKCs is a complex process. It starts with the maturation consisting of three strictly regulated phosphorylation steps, and subsequent recruitment to membrane through calcium or diacyl glycerol (DAG) dependent interactions. The final step in the activation of PKC ϵ is the interaction with DAG, and therefore incorporation to the membrane fraction in vivo can be used as a crude estimate of activity of this enzyme in vivo. In our experiments Clone A cells were either kept in suspension or plated on collagen or laminin-1 for 30-45 minutes. PKC α was not found in the membrane fraction in any of the conditions, whereas a pool of PKC ϵ was found associated with the membrane in all conditions tested. These results suggest that PKC ϵ is partly active in a constitutive manner, and the interaction with the extracellular matrix does not affect it.

We also tested the effect of brief treatment LY294002, a potent and specific inhibitor of PI3-K activity. This short treatment did not change the amount of PKC ϵ in membrane fraction. Although PI3-K activity is needed for the phosphorylation of PKC ϵ by PDK-1 (Cenni et al., 2002), this is a very early step in the activation of PKC ϵ , and therefore it is not surprising that short term inhibition of PI3-K does not cause any changes in the membrane association of PKC ϵ .

Morphology and video microscopy of Clone A cells was performed by Dr. Leslie M Shaw. Kinase inactive PKC ϵ caused a collapse of lamellae, and an increase in the number of retraction fibers suggestive of problems with detachment from the extracellular matrix. Kinase inactive PKC ζ did not cause any of these effects, which supports the hypothesis that PKC ϵ has a specific role in cell migration. In cells transiently transfected with kinase inactive PKC ϵ the lamellae initially form normally, but they are unstable and rapidly collapse. This collapse inhibits cell motility and prevents the cells from efficiently using their actin cytoskeleton for cell motility.

Previous studies from this group have suggested causal relationship between RhoA activation and induction of lamellae in Clone A cells (O'Connor and Mercurio, 2001; O'Connor et al., 2000). The relationship between PKC ϵ and RhoA activation is therefore especially interesting. PMA has been known for a long time to activate RhoA (Nishiyama et al., 1994), but the exact pathway responsible for activation is not known. On the other hand, our data so far demonstrates that a pool of PKC ϵ is constitutively in its membrane bound, activated form. We studied the activation of RhoA upon adhesion to laminin-1 using the well-characterized Rhotekin pulldown assay. Our data suggest that RhoA activity is at its highest 30 minutes after plating on laminin-1, and slowly decreases after that as previously described. PKC inhibitors (Go6976 and Go 6983) do not affect RhoA activation induced by interaction with laminin-1. In cells plated on collagen for two hours the RhoA activity is extremely low. In these cells RhoA can be rapidly activated by treatment with PMA. Activation of RhoA by PMA is completely blocked by broad spectrum PKC inhibitor Go6983. We therefore conclude that activation of RhoA upon adhesion to laminin-1 is independent of PKC activity, and PKC ϵ creates a separate, parallel signal that facilitates cell migration.

As the final step, we studied these signaling pathways in carcinoma cell invasion. MDA-MB-435 cells were used in Matrigel invasion assays as previously described. Kinase inactive PKC ϵ reduced the invasion by 50%, whereas kinase inactive PKC ζ had no effect.

Key research accomplishments

The results of the studies on PKC ϵ in carcinoma cell migration and invasion can be summarized as follows:

1. PKC ϵ is constitutively active in carcinoma cells. Because the early steps of PKC ϵ activation are PI3-K dependent, the $\alpha 6 \beta 4$ /PI3-K pathway facilitates PKC ϵ signaling in the long run.

2. Although a pool of PKC ϵ is in its active, membrane bound form in Clone A cells plated on collagen, these cells do not form lamellae because the initial signal for lamellae formation (RhoA activation) is missing
3. PMA treatment activates RhoA, which induces lamellae even when cells are plated on collagen.
4. PKC ϵ and RhoA act on separate, parallel pathways in facilitating carcinoma cell migration and invasion.
5. RhoA is the initial signal for induction of lamellae, whereas PKC ϵ stabilizes the lamellae and enables efficient use of actin cytoskeleton for cell motility.
6. Inhibition of PKC ϵ activity reduces breast carcinoma cell invasion.

Reportable outcomes

Tani, T.T., and A.M. Mercurio. 2001. PDZ Interaction Sites in Integrin alpha Subunits. TIP-2/GIPC BINDS TO A TYPE I RECOGNITION SEQUENCE IN alpha 6A/alpha 5 AND A NOVEL SEQUENCE IN alpha 6B. *J Biol Chem.* 276:36535-36542.

Conclusions

Our previous studies have demonstrated the importance of $\alpha 6\beta 4$ integrin for breast carcinoma invasion. In this project we have described one more pathway that operates downstream of $\alpha 6\beta 4$ /PI3-K pathway. The fact that PKC ϵ has a specific role in breast carcinoma invasion provides a target for pharmacological and gene therapy in advanced breast cancer. Although PKC isoforms are highly similar in their structure, our work demonstrates that it is possible to find specific roles for each of them. PKC ϵ is a promising target for future research on the regulation of actin cytoskeleton in invasive breast carcinoma cells.

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