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Association of plakoglobin with APC, a tumor suppressor gene product, and its regulation by tyrosine phosphorylation

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Plakoglobin is a cytoplasmic protein localized in both adherens junctions and desmosomes. Little is known about its function, but it may play a role in maintaining cell junction integrity. A partly homologous protein, β catenin, is localized mainly in adherens junctions and plays a key role in cell adhesion by associating with cadherins, a family of Ca^{2+} dependent cell-to-cell adhesion molecules. Recently the product of APC, a tumor suppressor gene, was found to associate with β catenin. In this study we demonstrated that plakoglobin also associates with APC and that tyrosine phosphorylated plakoglobin associates with cadherins but not with APC. These results suggest that plakoglobin could play a role in mediating the signals of APC by mutual interaction and that this may be regulated by tyrosine phosphorylation.

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Cell-cell interaction mediated by cell adhesion plays an important role in cell development, differentiation and carcinogenesis (1). The major adhesion apparatus present in epithelial cells are adherens junctions and desmosomes.

Plakoglobin, an 83-kDa cytoplasmic protein, isolated from the desmosomal fraction (2), shares partial amino acid similarity with β catenin (3). It exists in both desmosomes and adherens junctions and associates with desmoglein and cadherins (4, 5). Thus plakoglobin may be a common regulatory molecule in cell junctions.

It is reported that cell adhesion mediated by cadherins is regulated by tyrosine phosphorylation of β catenin (6,7). Tyrosine phosphorylation of these cytoplasmic proteins interacting with cell adhesion molecules seems to regulate the cell adhesion system.

Recently APC (adenomatous polyposis coli), the product of a tumor suppressor gene of colon cancer, was found to associate with β catenin (8, 9), suggesting that cytoplasmic proteins like catenins may regulate APC function as well as cell adhesion.

To understand the signal pathway mediated by plakoglobin, we attempted to isolate proteins interacting with plakoglobin using the Far-Western method. In the course of screening, we found that APC also associates with plakoglobin as well as β catenin.

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Materials and methods

Cell lines used in this study were A-431, a human squamous cell carcinoma of the vulva, and Okajima, a human poorly differentiated adenocarcinoma of the stomach. Monoclonal antibodies against APC (Oncogene Science), plakoglobin (Cymbus), phosphotyrosine (Seikagaku Kougyo) were obtained commercially, monoclonal antibody against E-cadherin, HECD-1, has been described previously (10).

The Far-Western method was performed as described previously (9, 11). Briefly, full-length human plakoglobin cDNA (12) was subcloned in pGEX-2TK (Pharmacia). The recombinant protein was purified and labeled with [^{32}P]ATP as described (9). A human brain cDNA library (Clonetech) was screened, and isolated clones were subcloned into pBluescript SK- (Strategene) and sequenced by the dideoxynucleotide chain termination method (USB).

Protein-affinity chromatography was performed as follows. Isolated cDNA fragments of APC and N-cadherin (a member of the cadherin family expressed in brain) were subcloned into pGEX-4T1 (Pharmacia), and recombinant GST fusion proteins were prepared and purified using Sepharose 4B (Pharmacia). Cell lysates from A431 and Okajima were batch-absorbed to Sepharose beads coupled with the recombinant proteins. After five washings with lysis buffer, the eluates were fractionated by SDS-PAGE and the immunoblots were developed with anti-plakoglobin antibody.

Immunoprecipitation and immunoblotting were performed as described previously (10).

Results

Plakoglobin interaction with APC

To isolate proteins interacting with plakoglobin *in vitro*, we performed the Far-Western method. Using recombinant plakoglobin as a probe, we screened about $5x10^5$ clones of a human brain λgt11 cDNA expression library. Several clones were isolated and sequenced. There were partial in-frame clones of APC and N-cadherin. The shortest APC clone encoded amino acids 725-1089. This fragment was used for protein-affinity chromatography. All of the isolated N-cadherin clones contained the intracellular domain, and one of them which encoded amino acids 586-906 was examined further.

Next we attempted to demonstrate protein-protein interaction *in vitro* by protein-affinity chromatography. As shown in figure 1, plakoglobin was recovered from GST-APC (lanes 3,6) and GST-N-cadherin (lanes 2,5), but none from GST only (lanes 1,4).

In order to investigate whether plakoglobin associates with APC *in vivo*, the cell lines A431 and Okajima were lysed and immunoprecitation with anti-APC antibody was performed. As shown in figure 2, plakoglobin was detected in the APC immunoprecipitates.

Association of phosphorylated plakoglobin with E-cadherin but not with APC

As plakoglobin exists in both plaque-bound and soluble forms (2), certain factors determine its localization or association status. Post-translational modification of plakoglobin may affect its association affinity for cadherin and APC. In order to examine tyrosine-phosphorylated plakoglobin *in vivo*, we used Okajima cells, which express E-cadherin and show extensive tyrosine phosphorylation of plakoglobin (T.S., A.O., and S.H., unpublished data). Immunoprecipitates were obtained with antibodies against E-cadherin (Fig.3,lane 1) and APC (Fig.3,lane 2), and these were immunoblotted with antibodies against plakoglobin and phosphotyrosine. Tyrosine-phosphorylated plakoglobin was not detected in the immunoprecipitates with anti-APC antibody (Fig.3 lane,2). On the other

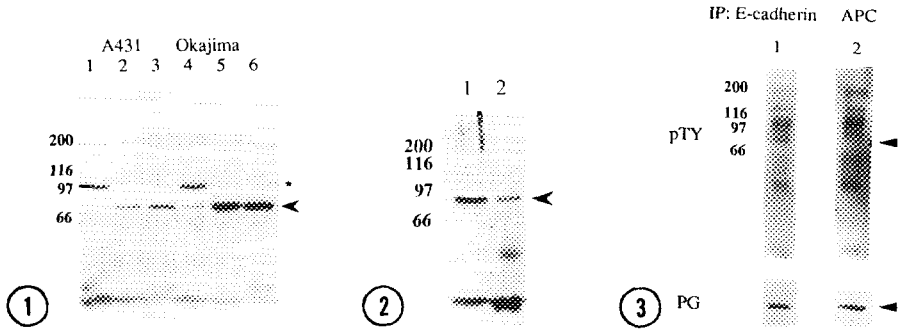

Figure 1. **Protein-affinity chromatography of plakoglobin and GST fusion proteins.** Cell lysates of A431 (lanes 1, 2, 3) and Okajima (lanes 4, 5, 6) were adsorbed on Sepharose beads coupled with GST (lanes 1, 4), GST-N-cadherin (lanes 2, 5), and GST- APC (lanes 3, 6). The eluates were fractionated by SDS-PAGE and bound plakoglobin was detected by immunoblotting. Plakoglobin is indicated by the arrowhead. The asterisk indicates a non-specific band. Molecular weight markers are indicated on the left.

Figure 2. **Immunoblot analysis of immunoprecipitates from A431 (lane 1) and Okajima (lane 2) obtained with the antibody against APC.** The immunoblot was developed with anti-plakoglobin antibody. Plakoglobin is indicated by the arrowhead. Molecular weight markers are indicated on the left.

Figure 3. **Immunoblot analysis of E cadherin and APC immunoprecipitates.** Immunoprecipitates obtained with anti-E cadherin antibody (lane 1) and anti-APC antibody (lane 2) from Okajima cells were subjected to immunoblot analysis for detection of phosphotyrosine (upper panel) and plakoglobin (lower panel). Plakoglobin is indicated by the arrowhead. Molecular weight markers are indicated on the left.

hand, immunoprecipitates with anti-E-cadherin antibody contained tyrosine-phosphorylated plakoglobin (Fig.3,lane 1), although about same amount of plakoglobin was present in the immunoprecipitates.

Discussion

The Far-Western method, protein-affinity chromatography and immunoprecipitation revealed that plakoglobin associated with APC *in vivo* and *in vitro*. Thus plakoglobin like β catenin, associates with APC. The shortest fragments of APC cDNA isolated by the Far-Western method included the β catenin binding domain (9). Therefore we consider that β catenin and plakoglobin share the same binding domain of APC.

Although the function of APC is still unknown, the present results imply that it may interact with cell adhesion regulators of both adherens junctions and desmosomes. It is reported that genetic aberration of APC occurs in adenoma, an early stage of colon carcinogenesis (13). As disorganization of colon epithelial cells is found in adenoma, cell polarity or contact inhibition regulated by cell adhesion might be the primary target of colon carcinogenesis.

Because cadherins are localized in the cell junction and APC exists in the cytoplasm (14), we attempted to clarify the mechanism determining the localization of plakoglobin. In

mammalian cells, aberrant activation of src, a non-receptor tyrosine kinase, causes a drastic change of cell shape and cell adhesion (6,7). In these cells, abnormal accumulation of tyrosine-phosphorylated proteins including β catenin has been reported. Therefore tyrosine phosphorylation seems to be one of the mechanisms regulating the function of these proteins. Accordingly, we compared the tyrosine phosphorylation status of the cadherin-bound and APC-bound forms of plakoglobin. Surprisingly, APC did not associate with tyrosine-phophorylated plakoglobin. Therefore tyrosine-phosphorylated plakoglobin may bind preferentially to cadherins and thus become localized in adherens junctions. Some tyrosine kinases, such as src, may regulate associations among APC, cadherins and plakoglobin.

In conclusion, we have found that plakoglobin associates with APC and that this association may be regulated by tyrosine phosphorylation. These results suggest that plakoglobin and APC may work cooperatively to regulate cell adhesion and cell growth.

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