Roles of plakoglobin end domains in desmosome assembly

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SUMMARY

Plakoglobin, a member of the armadillo family of proteins, is a component of intercellular adhesive junctions. The central domain of plakoglobin comprises a highly conserved series of armadillo repeats that facilitate its association with either desmosomal or classic cadherins, or with cytosolic proteins such as the tumor suppressor gene product adenomatous polyposis coli. Sequences in the N- and C-terminal domains of plakoglobin are less highly conserved, and their possible roles in regulating plakoglobin’s subcellular distribution and junction assembly are still unclear. Here we have examined the role of plakoglobin end domains by stably expressing constructs lacking the N and/or C terminus of plakoglobin in A-431 cells. Our results demonstrate that myc-tagged plakoglobin lacking either end domain is still able to associate with the desmosomal cadherin desmoglein and incorporate into desmosomes. In cell lines that express an N-terminal truncation of plakoglobin, an increase in the cytosolic pool of endogenous and ectopic plakoglobin was observed that may reflect an increase in the stability of the protein. Deletion of the N terminus did not have a dramatic effect on the structure of desmosomes in these cells. On the other hand, striking alterations in desmosome morphology were observed in cells expressing C-terminal truncations of plakoglobin. In these cell lines, ectopic plakoglobin incorporated into desmosomes, and extremely long junctions or groups of tandemly linked desmosomes which remained well attached to keratin intermediate filaments, were observed. Together, these results suggest that plakoglobin end domains play a role in regulating its subcellular distribution, and that the presence of the C terminus limits the size of desmosomes, perhaps through regulating protein-protein interactions required for assembly of the desmosomal plaque.

Key words: Plakoglobin, Armadillo family, Desmosome

INTRODUCTION

The junction associated protein plakoglobin (Pg) is a member of the growing armadillo family of proteins which includes the adherens junction protein β-catenin and its Drosophila homologue, the segment polarity gene product Armadillo (Peifer et al., 1994a). β-catenin was first recognized as a cell junction protein by virtue of its localization to adherens junctions, where it serves as a bridge linking classical cadherins to α-catenin (Aberle et al., 1996b). α-catenin in turn interacts directly or indirectly with the actin cytoskeleton (Knudsen et al., 1995; Rimm et al., 1995). However, in recent years it has been realized that, like Armadillo, which was originally defined as a downstream effector of the Drosophila wingless signaling pathway, β-catenin also plays a more global role in intracellular signaling pathways and may even act as a transcriptional transactivator (for reviews see Gumbiner, 1995; Huber et al., 1996; Miller and Moon, 1996; Peifer, 1995).

Plakoglobin, a close relative of β-catenin, has been reported as a constituent both of desmosomes, adhesive junctions that provide cell surface attachment sites for intermediate filaments (IF), and adherens junctions (Cowin, 1994; Cowin and Burke, 1996; Cowin et al., 1986). Although β-catenin’s contribution to junction structure and adhesive function as well as signal transduction has been firmly established, the role of Pg in intercellular junctions and signaling is not as well understood. With regard to signaling, like β-catenin, Pg can promote axis duplication in Xenopus (Karnovsky and Klymkowsky, 1995). In addition, a structural role for this molecule in recruiting IF to the desmosomal plaque has been suggested (Troyanovsky et al., 1994b). As Pg preferentially incorporates into desmosomes in cells that assemble both adherens junctions and desmosomes (Adams et al., 1996; Nathke et al., 1994) this molecule may play a particularly important role in cytoskeletal attachment and adhesive function in these junctions. Finally, Pg knock out mice have recently been shown to exhibit an embryonic lethal phenotype due to devastating structural defects of intercalated discs in the developing heart muscle (Bierkamp et al., 1996; Ruiz et al., 1996).

The functions of armadillo family members are dependent on their interactions with a diverse group of partner proteins present in different subcellular compartments. So far these protein interactions appear to be mediated in large part by overlapping regions of the central armadillo repeats for which the family is named. Plakoglobin itself interacts with the cytoplasmic tails of the desmosomal cadherins, the desmogleins (Dsg) and the desmocollins (Dsc) (Korman et al., 1989;
Kowalczyk et al., 1994; Mathur et al., 1994; Peifer et al., 1992; Roh and Stanley, 1995; Troyanovsky et al., 1994a,b), as well as classical cadherins (Butz and Kemler, 1994; Jou et al., 1995; Knudsen and Wheelock, 1992; Peifer et al., 1992; Piepenhagen and Nelson, 1993). Plakoglobin also interacts with cytoplasmic proteins such as α-catenin (Aberle et al., 1994; Hulsken et al., 1994; Rubinfeld et al., 1995) and the tumor suppressor gene product adenomatous polyposis coli (APC) (Shibata et al., 1994). More centrally located armadillo repeats are required for association with classical cadherins, whereas the repeats flanking the central region are essential for desmosomal cadherin binding (Chitaev et al., 1996; Ozawa et al., 1995; Sacco et al., 1995; Troyanovsky et al., 1996; Wahl et al., 1996; Witcher et al., 1996). In particular, Dsg binding to Pg is dependent on the integrity of sequences in repeats 1-3, a site that partially overlaps with the region required for α-catenin binding (Aberle et al., 1996a; Ozawa et al., 1995; Sacco et al., 1995; Troyanovsky et al., 1996; Wahl et al., 1996; Witcher et al., 1996). This overlap in binding domains of desmosomal cadherins and α-catenin may be important in regulating the cytoskeletal linkages associated with desmosomal versus classical cadherins.

Although Pg, β-catenin and Armadillo share a high degree of sequence similarity in their central domains, the N- and C termini of the family members are more divergent (Peifer et al., 1992; Peifer and Wieschaus, 1990). This sequence divergence suggests that the end domains may confer specific regulatory functions on the armadillo family members. We have addressed the functions of Pg end domains by generating stable A-431 cell lines expressing full length myc tagged Pg or truncations lacking the N terminus (PgΔN), C terminus (PgΔC) or both N- and C termini (PgΔNΔC) (Fig. 1). Our results suggest that Pg end domains contain regulatory sequences that govern the balance of Pg in various subcellular pools and also affect the assembly of intercellular junctions. In particular, the Pg C terminus appears to limit the length of desmosomes, perhaps by regulating the extent of protein-protein interactions that give rise to the desmosomal plaque.

Fig. 1. Schematic representation of Pg constructs. Pg constructs encoding full length Pg (Pg), an N-terminal truncated Pg (PgΔN), a C-terminal truncated Pg (PgΔC) and an N and C-terminal truncated Pg (PgΔNΔC) are shown. The shaded boxes represent the armadillo repeats found in Pg and its family members (Peifer et al., 1994a). The numbers designate amino acids expressed for each construct. Constructs are myc epitope tagged.

**MATERIALS AND METHODS**

**Generation of plakoglobin cDNA constructs**

Full length human Pg cDNA was isolated and subcloned into the mammalian expression vector LK 444 under the control of the human β-actin promoter (Kowalczyk et al., 1994). The nucleotide numbers listed for all Pg constructs listed below correspond to the human Pg sequence submitted to GenBank (accession number Z68228; Franke et al., 1989).

An N-terminal deletion of Pg was generated using the previously described Pg construct p236 (Kowalczyk et al., 1994) as a PCR template and primers LN123 (5’ACGGTGTCAGCAAGCAGGATGTCGAGAACCCTGAAGAAGC-AGAGCTTCG) and LN52 (5’TCGAGAGCACCACATGTGC-GCCGTCGACCAGAAGCTCATTTCTGAAGAGGACTTGAGAAGCTTGCC). The resulting PCR product, PgΔN (nucleotides 486-2354), contains a 5′ SalI restriction site and Kozak consensus sequence at the 5′ end, and an 11 amino acid c-myc epitope tag and a HindIII restriction site at the 3′ end. Using the engineered restriction sites, tagged PgΔN was subcloned into the SalI/HindIII site in pBluescript. To ensure that the Pg sequence was correct, the resulting plasmid, p464, was digested with SstI (nucleotide 598) and BglII (nucleotide 1855) and replaced with a SstIBglII fragment from p236. In addition, the remaining PCR generated ends of p464 were sequenced. Using the SalI/HindIII sites in p464, PgΔN was subcloned into the SalI/HindIII sites of LK 444 generating plasmid p465. p465 was used as an expression vector with the PgΔN cDNA expression driven by the β-actin promoter.

A C-terminal deletion of Pg, which also removes a small portion of the 13th armadillo repeat, was generated using p236 as a PCR template and primers LN51 (5’ACGGTGTCAGCAAGCAGGATGTCGAGAACCCTGAAGAAGC-AGAGCTTCG) and LN120 (5’TCTGAGAGCACCACATGTGC-GCCGTCGACCAGAAGCTCATTTCTGAAGAGGACTTGAGAAGCTTGCC). The resulting PCR product, PgΔC (nucleotides 2360-1995), is used as an expression vector with the PgΔC cDNA expression driven by the β-actin promoter.

**Stable cell lines**

A-431 epithelial cells (a gift from Dr M. Wheelock, University of Toledo) were cultured in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. cDNA constructs encoding full length Pg (p330), PgΔN (p465), PgΔC (p511) and PgΔNΔC (p468), with the neomycin resistance gene on the same
plasmid, were transfected into A-431 cultures in duplicate as described previously (Bornslaeger et al., 1996). Individual clones of each cell line were isolated and maintained in medium containing 350 µg/ml (active concentration) G418. Expression of Pg proteins in the cell lines was assayed by SDS-PAGE (6-12% acrylamide gradient gel) and immunoblot analysis.

Densitometric analysis

Using the Bio-Rad model GS-670 imaging densitometer and the Molecular Analyst image analysis software, 2-5 immunoblots of whole cell extracts, probed with Pg specific antibodies, were analyzed to determine the levels of endogenous and ectopic Pg in the Pg cell lines. Once the total amount of Pg (endogenous + ectopic) in the cell lines was determined, the percentage endogenous versus ectopic Pg was calculated. As the Pg specific antibodies used in the immunoblots do not recognize PgAN/AC an indirect comparison of Pg to PgAN/AC was made by first determining the ratio of PgAN/AC detected by the N-terminal Pg specific antibody compared to the PgAN/AC detected by the myc antibody. This ratio was then used to generate an estimate for PgAN/AC, based on the amount of myc reactive PgAN/AC.

Antibodies

A rabbit polyclonal antibody directed against the c-myc epitope, was a gift from Dr J. Stanley (University of Pennsylvania). The following antibodies were previously described: the mouse monoclonal antibody against the c-myc epitope tag, 9E10.2 (Evans et al., 1985), the mouse monoclonal antibody 11E4, directed against the N terminus of plakoglobin (Kowalczyk et al., 1994), a rabbit polyclonal antibody directed against the C terminus of plakoglobin (Hinck et al., 1994), the rabbit polyclonal antibody NW6, directed against desmoplakin (Angst et al., 1990), the mouse monoclonal antibody 6D8, directed against Dsg2 (Bornslaeger et al., 1996; Wahl et al., 1996), the mouse monoclonal antibody IG5, directed against a-catenin (Johnson et al., 1993). 11E4, 6D8 and IG5 were provided by Dr M. Wheelock (University of Toledo) and the Pg C-terminal antibody was provided by Dr J. Papkoff (Megabios Corp., Burlington, CA). The mouse monoclonal anti-cytokeratin peptide 18 antibody (KSB17.2) was purchased from Sigma (St Louis, Missouri). For immunoblotting the polyclonal antibody directed against the c-myc tag was used at 1:500, concentrated 11E4 supernatant at 1:2,000, the polyclonal antibody against the Pg C terminus at 1:4,000, and 6D8 ascites at 1:1,000. Primary antibodies were diluted in PBS, 5% milk. Secondary antibodies, goat anti-mouse peroxidase and goat anti-rabbit peroxidase (Kirkegaard & Perry Laboratories, Gaithsburg, MD), were used at a dilution of 1:5,000 in PBS, 5% milk. Antibodies were detected using Enhanced Chemiluminescence (Amersham).

Sequential detergent extraction

Cells were grown to confluence on 60 mm culture dishes, rinsed in complete PBS and scraped into 150 µl cold saponin buffer (0.01% saponin, 10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 5 mM EDTA, 2 mM EGTA, 1 mM PMSF). Proteins were extracted for 20 minutes on ice and samples centrifuged at approximately 14,000 g for 30 minutes at 4°C. After centrifugation, the saponin soluble pool was transferred to a fresh tube and 50 µl reducing SDS-PAGE sample buffer was added. The remaining pellet was extracted in 150 µl cold Triton X-100 buffer (1% Triton X-100, 10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 5 mM EDTA, 2 mM EGTA, 1 mM PMSF). Samples were vortexed for 1 minute and centrifuged at approximately 14,000 g for 30 minutes at 4°C. The Triton X-100 soluble pool was transferred to a fresh tube and 50 µl reducing SDS-PAGE sample buffer was added. The Triton X-100 insoluble proteins were solubilized in 200 µl SDS/urea buffer (1% SDS, 8 M urea, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 2 mM EGTA, 1 mM PMSF). The saponin and Triton X-100 soluble samples were heated at 95°C for 10 minutes and all the samples were centrifuged at approximately 14,000 g for 5 minutes and subjected to SDS-PAGE on a 6-12% gradient gel. Proteins were transferred to nitrocellulose and immunoblot analysis was performed. Densitometric analysis using the Bio-Rad model GS-670 imaging densitometer and the Molecular Analyst image analysis software was performed for three independent experiments to determine the distribution of endogenous and ectopic Pg among the three pools.

Immunoprecipitation

For immunoprecipitations from the saponin pool, cells were grown on 60 mm culture dishes, rinsed in complete PBS and scraped into 1 ml cold saponin buffer (see above). Proteins were extracted for 20 minutes on ice and samples centrifuged at approximately 14,000 g for 30 minutes at 4°C. For immunoprecipitations from the Triton X-100 pool, cells were grown on 100 mm culture dishes, rinsed in complete PBS and scraped into 1 ml cold Triton X-100 buffer (see above). Samples were vortexed for 1 minute and centrifuged at approximately 14,000 g for 30 minutes at 4°C. Samples were preclared with 50 µl Gamma Bind Plus Sepharose beads (Pharmacia, Uppsala, Sweden) for at least 1 hour at 4°C and centrifuged for 5 minutes to remove non-specific complexes. Protein complexes from the saponin pool were immunoprecipitated using the polyclonal myc antibody and Triton X-100 soluble complexes were immunoprecipitated using the antibody 6D8, directed against Dsg2. Antibodies were added for one hour after which 50 µl Gamma Bind Plus Sepharose beads were added for an additional hour. Beads were washed 4 times each with 1 ml 1% Triton X-100 wash buffer, resuspended in 70 µl reducing SDS-PAGE sample buffer and heated at 95°C for 10 minutes. Samples were subjected to SDS-PAGE on a 7.5% gel and immunoblot analysis.

Immunofluorescence

Cells were grown on coverslips in 35 mm culture dishes, rinsed in PBS and fixed in cold methanol for 2 minutes. To detect ectopic Pg, concentrated supernatant of the monoclonal antibody against the c-myc tag, 9E10, was used at a 1:10 dilution or, the polyclonal antibody against the c-myc tag was used at a 1:900 dilution. 9E10 staining was detected using a rhodamine-conjugated goat anti-mouse IgG and the polyclonal myc tag antibody was detected using a rhodamine-conjugated goat anti-rabbit IgG. Endogenous Pg was detected using concentrated supernatant of the Pg specific monoclonal antibody 11E4 diluted 1:30 in PBS. 11E4 staining was detected using a fluorescein-conjugated goat anti-mouse IgG. Desmoplakin protein was detected using the polyclonal antibody NW6 diluted 1:50 in PBS. NW6 staining was detected using a fluorescein-conjugated goat anti-rabbit IgG. Keratin filaments were detected using the monoclonal antibody KSB17.2 diluted 1:200 in PBS. The keratin antibody was detected using a rhodamine-conjugated goat anti-mouse IgG. a-catenin was detected using the monoclonal antibody IG5 diluted 1:1 in PBS. IG5 staining was detected using a rhodamine-conjugated goat anti-mouse IgG. All secondary antibodies were purchased from Kirkegaard & Perry Laboratories, Gaithsburg, MD and diluted 1:50 in PBS.

Electron microscopy

Cells were grown to confluence on 60 mm culture dishes and processed for conventional electron microscopy as described previously (Green et al., 1991). Briefly, cells were fixed in 1% glutaraldehyde and post-fixed in 1% osmium tetroxide. Samples were stained with aqueous 1% uranyl acetate, dehydrated in a graded ethanol series and embedded in epon-Araldite. Ultra-thin sections were viewed using a JEOL 100CX.

RESULTS

Generation of stable cell lines expressing myc-tagged full length Pg or Pg lacking one or both domains

To address the function of Pg end domains in the subcellular
distribution of Pg and in intercellular junction assembly. A-431 cell lines expressing myc epitope tagged PgΔN, PgΔC and PgΔN/ΔC (Fig. 1) were generated. To determine if the myc tag affected the subcellular distribution of Pg or its incorporation into intercellular junctions, A-431 cell lines expressing a full length myc-tagged Pg construct (Pg.myc) were also generated. Each of the ectopically expressed proteins, detectable by Pg antibodies or the myc antibody, migrated at the predicted molecular mass (Fig. 2A,B). The level of ectopically expressed Pg.myc, PgΔN and PgΔC was directly compared to endogenous Pg levels by using Pg specific antibodies directed against the Pg N or C terminus (Fig. 2A). Because PgΔN/ΔC is not detected by the Pg specific antibodies the level of ectopically expressed PgΔN/ΔC was indirectly compared to endogenous Pg levels as described in Materials and Methods. Although the level of ectopic and endogenous Pg varied somewhat among the cell lines and among the clones of each cell line (Fig. 2C), the specific phenotypes observed for the different cell lines is unlikely to be solely dependent on expression level, since clones such as PgΔN (A7), PgΔC (10) and PgΔN/ΔC (A1), with comparable percents of ectopic Pg (57%, 51%, 54% of total Pg, respectively), exhibited dramatically different juncional phenotypes (see Fig. 6).

**The subcellular distribution of full length myc-tagged Pg is similar to endogenous Pg in control cells**

To determine any possible effects of the C-terminal myc tag on Pg, the subcellular distribution of full length myc-tagged Pg and endogenous Pg were compared both biochemically and morphologically in stable transfectants and control cells. In control A-431 cells endogenous Pg was distributed between a cytosolic saponin-soluble pool, a membrane bound Triton-soluble pool, and a Triton-insoluble pool, thought to be largely junction or cytoskeleton-associated (Fig. 3). Similar to endogenous Pg from control cells, a small proportion of full length myc-tagged Pg was observed in the cytosolic pool and the majority of the protein was in the Triton-soluble and Triton-insoluble pools (Fig. 3). Although there appeared to be a modest decrease in the amount of endogenous and ectopic Pg in the Triton-insoluble pool compared to control cells (Fig. 3), endogenous Pg and Pg.myc were nevertheless able to incorporate into junctions as assessed by co-localization with
endogenous cell junction components such as DP (see Fig. 6A,B), Dsg and Dsc (data not shown).

**Deletion of the Pg N terminus results in a larger cytosolic pool of ectopic and endogenous Pg, but neither end domain is required for the incorporation of Pg into a final Triton X-100 insoluble pool**

To determine the role of Pg end domains in the regulation of the subcellular distribution of the Pg protein, the distribution of both endogenous and ectopic Pg in cell lines expressing the various Pg truncations was analyzed. Unlike endogenous Pg in control cells or Pg.myc in which a small proportion of the total protein was in the cytosolic pool, a greater proportion of PgΔN and PgΔN/ΔC was observed in the cytosolic pool. Densitometric analysis of three independent extractions revealed only 14% of the total Pg in control cells, and 19% of Pg.myc, in the cytosolic pool. In contrast, 42% of PgΔN and 46% of PgΔN/ΔC was observed in the cytosolic pool. Interestingly, the differences in the subcellular distribution of PgΔN and PgΔN/ΔC were accompanied by similar alterations in the distribution of endogenous Pg in each of these A-431 cell lines. Endogenous Pg in the cytosolic pool accounted for 33% of total endogenous Pg in the mutant cell lines compared to 14% in control cells (Fig. 3). In addition to the differences seen in the cytosolic pool, there was a smaller proportion of endogenous Pg in the Triton-insoluble pool in PgΔN and PgΔN/ΔC cell lines. Nevertheless, deletion of the N terminus did not prevent ectopic or endogenous Pg in these cell lines from incorporating into intercellular junctions as assessed by immunofluorescence analysis (see Fig. 6).

Although the deletion of the Pg C terminus also resulted in an increase in the cytosolic pool of PgΔC compared to endogenous Pg in control cells, it was not as dramatic an increase as was seen for PgΔN or PgΔN/ΔC (Fig. 3). In addition, although there was an apparent decrease of endogenous Pg in the Triton-insoluble pool in PgΔC cell lines (Fig. 3), this decrease was not detectably different from that seen in full length Pg.myc cell lines. As in the case of PgΔN, deletion of the C terminus did not prevent PgΔC or endogenous Pg in these cell lines from incorporating into junctions (see Fig. 6).

To determine if the increase in the cytosolic pool of endogenous Pg seen in cell lines expressing N-terminal truncations of Pg was due to the formation of complexes between endogenous and ectopic Pg, cytosolic protein complexes were analyzed. A polyclonal antibody directed against the myc epitope tag co-immunoprecipitated endogenous Pg in cell lines expressing PgΔN, PgΔC and PgΔN/ΔC (Fig. 4). In cell lines expressing Pg.myc, endogenous Pg was barely detectable in the cytosolic protein complex precipitated with the myc antibody (Fig. 4).

**Pg lacking the N or C terminus is found in a complex with desmoglein**

The Triton X-100 soluble pool of proteins from the A-431 cell lines was analyzed to determine if the N and/or C termini affect the ability of Pg to interact with desmoglein 2 (Dsg2). An antibody directed against the Dsg2 extracellular domain co-immunoprecipitated both PgΔN and PgΔC (Fig. 5). Furthermore, the truncated Pg proteins did not prevent complexes from forming between endogenous Pg and Dsg since endogenous Pg in all of the cell lines was able to co-immunoprecipitate with Dsg. PgΔN and PgΔC also co-immunoprecipitated with desmocollin and classical cadherins (data not shown). In addition, data from our laboratory has shown that both PgΔN and PgΔC can co-immunoprecipitate with another desmoglein isoform, Dsg1, when they are co-expressed in mouse L-cell fibroblasts.
the various A-431 cell lines. Immunoprecipitates from each cell line were run, in duplicate, on SDS-PAGE gels, transferred to nitrocellulose and immunoblotted with the Pg N-terminal antibody or the Pg C-terminal antibody. Endogenous Pg was barely detectable in immunoprecipitates from cell lines expressing Pg.myc, however, endogenous Pg co-immunoprecipitated with the myc antibody in cell lines expressing PgΔN, PgΔC and PgΔN/ΔC. The A-431 clones shown are Pg.myc (B7), PgΔN (A7), PgΔC (10) and PgΔN/ΔC (A1).

(Kowalczyk et al., 1997). Because the IgG heavy chain used in the immunoprecipitations migrated at the same relative molecular mass as PgΔN/ΔC (~55 kDa), we were unable to confirm PgΔN/ΔC’s presence in a Dsg complex. However, as PgΔN/ΔC incorporated into junctions (see Fig. 6) it is likely to do so by associating with members of the cadherin family. The ability of PgΔN and PgΔC to co-immunoprecipitate with desmosomal cadherins is consistent with the results of others showing that Pg armadillo repeats, and not the end domains, are required for the interaction of Pg with both desmosomal and classical cadherins (Chitaev et al., 1996; Ozawa et al., 1995; Sacco et al., 1995; Troyanovsky et al., 1996; Wahl et al., 1996; Witcher et al., 1996).

**Fig. 4.** Co-immunoprecipitation of endogenous Pg with ectopic Pg. Using the myc polyclonal antibody, protein complexes were immunoprecipitated from the saponin soluble pool of proteins from the various A-431 cell lines. Immunoprecipitates from each cell line were run, in duplicate, on SDS-PAGE gels, transferred to nitrocellulose and immunoblotted with the Pg N-terminal antibody or the Pg C-terminal antibody. Endogenous Pg was barely detectable in immunoprecipitates from cell lines expressing Pg.myc, however, endogenous Pg co-immunoprecipitated with the myc antibody in cell lines expressing PgΔN, PgΔC and PgΔN/ΔC. The A-431 clones shown are Pg.myc (B7), PgΔN (A7), PgΔC (10) and PgΔN/ΔC (A1).

**Fig. 5.** Co-immunoprecipitation of ectopic Pg with Dsg2. Using the Dsg 2 specific antibody 6D8, protein complexes were immunoprecipitated from the Triton X-100 soluble pool of proteins from the various A-431 cell lines. Immunoprecipitates from each cell line were run, in triplicate, on SDS-PAGE gels, transferred to nitrocellulose and immunoblotted with the Dsg antibody 6D8, the Pg N-terminal antibody or the Pg C-terminal antibody. Pg.myc (open arrow), which migrates slower than endogenous Pg, is detected by both Pg antibodies. PgΔC (open arrowhead) and PgΔN (filled arrowhead) are detected by the Pg N or C-terminal antibodies, respectively. A non-specific band (open circle) is detected in all the immunoprecipitates, including those from control cells, with the Pg C-terminal antibody. The A-431 clones shown are Pg.myc (B7), PgΔN (A7), PgΔC (10) and PgΔN/ΔC (A1).

**Cells expressing C-terminal deletions of Pg exhibit a more continuous, less punctate, distribution of desmosomal components**

In order to assess the possible effects of removing the Pg end domains on desmosome assembly, indirect double label immunofluorescence using the 9E10 antibody directed against the myc tag and an antibody against the desmosomal plaque protein desmoplakin (DP) was performed. In cells expressing Pg.myc the ectopic protein exhibited a punctate staining pattern at cell-cell borders that co-localized with endogenous DP (Fig. 6A,B). The staining pattern for DP in Pg.myc cells was similar to the punctate staining pattern observed for DP in control A-431 cells (not shown). Similar to Pg.myc, PgΔN exhibited a punctate staining pattern at cell-cell borders that co-localized with DP staining (Fig. 6C,D). In addition, a cytoplasmic myc staining pattern could be detected that did not always co-localize with DP, which may reflect in part the cytosolic pool of PgΔN observed biochemically (see Fig. 3).

In cell lines expressing PgΔC, the ectopic protein was distributed more continuously at some borders and DP in these cells had a more continuous staining pattern (Fig. 6E,F) compared to control or Pg.myc cells. The altered distribution of desmosomal components was most dramatic in cell lines expressing PgΔN/ΔC in which PgΔN/ΔC and DP frequently appeared more continuous at cell-cell borders (Fig. 6G,H). In addition to DP, other desmosomal components, such as Dsg and Dsc, exhibited a more continuous staining pattern in cell lines expressing C-terminal truncations of Pg (data not shown).

To determine if the phenotype in cell lines expressing C-terminal truncated Pg was in part due to the exclusion of endogenous Pg from these long junctions, indirect double label immunofluorescence using an antibody against the myc epitope tag and a Pg specific antibody recognizing only endogenous Pg was performed. In cell lines expressing PgΔN/ΔC, endogenous Pg exhibited a more continuous staining pattern in areas that co-localized with ectopic Pg staining (Fig. 7A,B). Endogenous Pg appeared more punctate in areas where ectopic Pg staining was barely detectable.

**Expression of Pg truncations does not disrupt intermediate filament attachment to the cell surface or induce mixing of desmosomal and adherens junction components**

Although Pg has been implicated in playing a role in recruit-
ing intermediate filaments (IF) to the desmosomal plaque (Troyanovsky et al., 1994b), how Pg contributes to IF attachment at the molecular level is not understood. To determine whether truncation of either the Pg N or C terminus compromised IF attachment to the cell surface, double label immunofluorescence using an antibody against DP and an antibody against keratin IF was performed. In all the cell lines expressing ectopic Pg proteins, IF bundles remained associated with the cell surface and terminated at sites of DP staining (Fig. 8).

In addition to playing a role in recruiting IF to the desmosomal plaque, Pg may also be involved in the segregation of junctional components. Junctions in the hearts of Pg null mice contain components of both desmosomes and adherens junctions suggesting that Pg may be involved in the sorting of desmosomal and adherens junction proteins (Ruiz et al., 1996). To determine if the N and/or C terminus of Pg is involved in this segregation, double label immunofluorescence using an antibody against DP and an antibody against the adherens

![Fig. 6. Ectopic Pg co-localizes with DP. Indirect double label immunofluorescence was performed to detect ectopic Pg polypeptides and endogenous DP. The localization of ectopic Pg in A-431 clones Pg myc (B7) (A and B), PgΔN (A7) (C and D), PgΔC (B7) (E and F) and PgΔN/ΔC (A1) (G and H) was determined by using the myc antibody 9E10 (A,C,E,G). DP in these cells was detected using the DP antibody NW6 (B,D,F,H). Note the more continuous staining pattern of DP in cell lines expressing Pg protein lacking the C terminus (F,H). All panels are at the same magnification. Bar, 10 μm.](image-url)
myc tag (A) and endogenous Pg was detected using the Pg N-terminal (A1). Ectopic Pg was detected using a polyclonal antibody against the antibody 11E4 (B). Note the co-localization of ectopic and endogenous immunofluorescence was performed on the A-431 clone PgΔN/ΔC (A1). Ectopic Pg was detected using a polyclonal antibody against the myc tag (A) and endogenous Pg was detected using the Pg N-terminal antibody 11E4 (B). Note the co-localization of ectopic and endogenous Pg in the extended junctions, but not in the normal punctate structures. Both panels are at the same magnification. Bar, 10 μm.

Fig. 7. Ectopic Pg co-localizes with endogenous Pg. To compare the localization of endogenous Pg and PgΔN/ΔC, double label immunofluorescence was performed on the A-431 clone PgΔN/ΔC (A1). Ectopic Pg was detected using a polyclonal antibody against the myc tag (A) and endogenous Pg was detected using the Pg N-terminal antibody 11E4 (B). Note the co-localization of ectopic and endogenous Pg in the extended junctions, but not in the normal punctate structures. Both panels are at the same magnification. Bar, 10 μm.

junction protein α-catenin was performed. DP and α-catenin exhibited distinct staining patterns in control cells and in all of the cell lines expressing ectopic Pg (Fig. 9).

Longer desmosomes and groups of tandemly linked desmosomes are present in cell lines expressing C-terminal deletions of Pg

Ultrastructural analysis was performed to determine if the continuous staining pattern observed by immunofluorescence in PgΔC and PgΔN/ΔC cell lines represented continuous desmosomes. Desmosomes observed in cell lines expressing PgΔN (Fig. 10B) were similar to desmosomes observed in control cells (Fig. 10A) and cell lines expressing Pg.myc (data not shown). However, in cell lines expressing PgΔC and PgΔN/ΔC many desmosomes appeared to occupy more of the plasma membrane and groups of tandemly linked desmosomes were observed (Fig. 10C,D). Consistent with the immunofluorescence analysis, IF attachment to the cell surface was not disrupted in any of the cell lines expressing ectopic Pg proteins.

DISCUSSION

Plakoglobin’s ability to interact with junctional proteins such as desmosomal cadherins, classical cadherins and α-catenin is dependent largely on the highly conserved armadillo repeats found in Pg and its family members (Chitaev et al., 1996; Ozawa et al., 1995; Sacco et al., 1995; Troyanovsky et al., 1996; Wahl et al., 1996; Witcher et al., 1996). Less is known regarding the function of the more divergent end domains of Pg, and their possible roles in regulating Pg’s association with various proteins or assembly into intercellular junctions. Here we demonstrate by stably expressing Pg with deletions of one or both end domains, that these domains contain information that regulates Pg’s subcellular distribution as well as normal assembly of the desmosomal plaque.

Deletion of the Pg N terminus led to an accumulation of both ectopic and endogenous Pg in the cytosolic pool (Fig. 3). Previously, our laboratory has shown that Pg is unstable and rapidly degraded when expressed in L-cell fibroblasts in the absence of a desmosomal cadherin (Kowalczyk et al., 1993). It is possible that accumulation of Pg lacking the N terminus in the cytosol reflects an increase in the stability of PgΔN and PgΔN/ΔC in a non-cadherin associated pool. The stability of the Pg family member β-catenin is dependent upon a glycogen synthase kinase 3 (GSK3) consensus site in the N terminus of β-catenin, and mutations or deletions of the N-terminal GSK3 site in β-catenin result in an increased stability of β-catenin (Barth et al., 1997; Munemitsu et al., 1996; Yost et al., 1996). A similar GSK3 consensus site is found in the N terminus of Pg (Peifer et al., 1994b) and deletion of this site results in the accumulation of Pg in Xenopus embryos (Rubenstein et al., 1997). Intriguingly, the expression of N-terminal truncations of Pg in A-431 cells resulted in an increase in the level of endogenous Pg in the cytosolic pool (Fig. 3). Previous investigators have suggested that Pg may be able to self-associate (Kapprell et al., 1987). If N-terminally truncated, stable Pg and endogenous Pg formed a complex in the cytosolic pool, this could potentially explain the observed accumulation of endogenous Pg. Although we did not observe obvious interactions between endogenous Pg and Pg.myc, our results suggest that deletion of either end domain, and particularly the N terminus, greatly enhances complex formation between ectopic and endogenous Pg (Fig. 4). Therefore the increase in stability of N-terminally truncated constructs, along with their enhanced ability to interact with full length Pg, may lead to endogenous Pg’s stabilization.

Recently, Barth et al. (1997) have shown that the expression of N-terminal truncations of β-catenin affected the morphology and migration of MDCK cells. Although we have not observed obvious effects on A-431 cell behavior, further studies will be required to determine whether expression of Pg deletions affects cell adhesion, migration or morphology.

As might be predicted by previous studies that mapped desmosomal cadherin binding sites to the Pg armadillo repeats (Chitaev et al., 1996; Ozawa et al., 1995; Troyanovsky et al., 1996; Wahl et al., 1996; Witcher et al., 1996) and our own data indicating that PgΔN and PgΔC still associate with Dsg2 (Fig. 5), deletion of either Pg end domain did not prevent incorporation of Pg into desmosomes (Fig. 6). Decreases in the Triton-insoluble pool were observed (Fig. 3), but this was the case even in cells expressing full length myc-tagged Pg, suggesting that the observed decrease may reflect a modest inhibition of incorporation due to the myc epitope tag that is on the C terminus of each of the ectopic Pg proteins. In spite of the rel-
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At a relatively low level of protein that entered a Triton-insoluble pool, particularly in the case of PgΔΝ/ΔC, the observed effects on desmosomal plaque assembly were dramatic. In cell lines expressing PgΔC or PgΔΝ/ΔC desmosomes appeared much longer and tandemly linked desmosomes were observed (Figs 6 and 10). Although the underlying basis for this effect on desmosomes is not yet clear, one possibility is that deletion of the Pg C terminus may enhance interactions with other desmosomal components. Consistent with this idea is the reported identification of a stretch of ten amino acids in Pg armadillo repeat thirteen, the most C-terminal repeat, that interacts with and masks upstream cadherin binding sites within Pg itself (Troyanovsky et al., 1996). Although this stretch of amino acids is deleted in the C-terminal truncated Pg constructs used in the present study, we did not observe an increase in the interaction between PgΔC and the desmosomal cadherin Dsg2 (Fig. 5). Deletion of the end domains did, however, enhance the interaction between endogenous and ectopic Pg, possibly contributing to the enhanced desmosomal plaque assembly seen in PgΔC and PgΔΝ/ΔC cell lines.

Exposure of cryptic binding sites in the armadillo repeats might also enhance interactions with other plaque components. One candidate protein is the desmosomal component DP. DP is involved in anchoring IF to the cell surface via its C terminus (Bornslaeger et al., 1996; Kouklis et al., 1994; Stappenbeck et al., 1993; Stappenbeck and Green, 1992). Although the N

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**Fig. 8.** Expression of ectopic Pg does not disrupt IF attachment. Dual color double label immunofluorescence was performed on control cells (A) and A-431 clones Pg.myc (B7) (B), PgΔΝ (A7) (C), PgΔC (10) (D) and PgΔΝ/ΔC (A1) (E, F). The DP antibody, NW6, was detected using a fluorescein-conjugated secondary antibody while the keratin antibody, KSB17.2 (Sigma) was detected using a rhodamine-conjugated secondary antibody. In areas where DP and keratin filaments overlap the staining pattern appears yellow. All panels are at the same magnification. Bar, 10 μm.
terminus of DP is required for targeting the molecule to the plaque (Bornslaeger et al., 1996; Stappenbeck et al., 1993), the proteins with which this DP domain interacts directly at the cell surface have not been identified. Recent work from our laboratory suggests that the DP N terminus associates directly with Pg and clusters Pg-desmosomal cadherin complexes when co-expressed in L cell fibroblasts (A. P. Kowalczyk et al., 1997). Furthermore, the Pg end domains were not required for this association. These observations are consistent with the work presented here demonstrating that when the same Pg truncations were expressed in A-431 cells, the ectopic Pg was able to co-localize with DP (Fig. 6). Since expression of the truncated proteins did not disrupt IF attachment to the cell surface (Fig. 8) the Pg N and C termini are apparently not required for linking the DP-IF complex to the cell surface. In fact, if sequences in the Pg C terminus mask cryptic binding sites in the armadillo repeats that constitute the DP binding region, deletion of these sequences may enhance interactions with DP and could contribute to the enhanced plaque formation seen in this study.

Analysis of Pg null mice has revealed another possible function for Pg in proper junction assembly. Junctions in the hearts of Pg null mice contain components of both desmosomes and adherens junctions and embryos die due to heart defects (Ruiz et al., 1996). The end domains of Pg do not appear to be involved in the segregation of junctional compo-
Fig. 10. Ultrastructural analysis of desmosomes in cell lines expressing Pg truncations. Electron micrographs of control cells (A) and A-431 clones PgΔN (A7) (B), PgΔC (10) (C) and PgΔN/ΔC (A1) (D). Intermediate filament attachment is not disrupted in any of the Pg cell lines. Note the groups of tandemly linked desmosomes in cell lines expressing Pg lacking the C terminus (C,D). All panels are at the same magnification. Bar, 0.25 μm.
tems as we did not see any mixed junctions in cell lines expressing Pg truncations (Fig. 9). Although desmosomes were able to form in epithelial organs of Pg null mice the desmosomes were normal and mutant embryos that died around birth had skin blistering and subcorneal acantholysis suggesting that Pg is required for proper desmosome function (Bierkamp et al., 1996).

The Pg N terminus contains regulatory sequences that are involved in the distribution of Pg in various subcellular pools while the Pg C terminus is involved in the proper assembly of the desmosomal plaque. The C terminus of Pg is also the target of post-translational modifications that may further affect its regulatory activities. We have preliminary data suggesting that the C terminus of Pg contains sequences required for EGF-induced Pg tyrosine phosphorylation (H. L. Palka and K. J. Green, unpublished data). After EGF stimulation, tyrosine phosphorylated Pg is predominantly in the Triton X-100 soluble pool. Thus, phosphorylation of the C terminus of Pg may act to prevent incorporation of soluble Pg complexes into junctions, thereby inhibiting junction assembly. Further studies will be required to determine whether such phosphorylation prevents association of Pg with desmosomal plaque components such as DP, thereby modulating desmosome assembly.

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