

The Binding of Plakoglobin to Desmosomal Cadherins: Patterns of Binding Sites and Topogenic Potential

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Abstract. Plakoglobin is the only protein that occurs in the cytoplasmic plaques of all known adhering junctions and has been shown to be crucially involved in the formation and maintenance of desmosomes anchoring intermediate-sized filaments (IFs) by its interaction with the desmosomal cadherins, desmoglein (Dsg), and desmocollin (Dsc). This topogenic importance of plakoglobin is now directly shown in living cells as well as in binding assays *in vitro*. We show that, in transfected human A-431 carcinoma cells, a chimeric protein combining the vesicle-forming transmembrane glycoprotein synaptophysin, with the complete human plakoglobin sequence, is sorted to small vesicles many of which associate with desmosomal plaques and their attached IFs. Immunoprecipitation experiments have further revealed that the chimeric plakoglobin-containing transmembrane molecules of these vesicles are tightly bound to Dsg and Dsc but not to endogenous plakoglobin, thus demonstrating that the binding of plakoglobin to desmosomal cadherins does not require its soluble state and is strong enough to attach large

structures such as vesicles to desmosomes.

To identify the binding domains and the mechanisms involved in the interaction of plakoglobin with desmosomal cadherins, we have developed direct binding assays *in vitro* in which plakoglobin or parts thereof, produced by recombinant DNA technology in *E. coli*, are exposed to molecules containing the "C-domains" of several cadherins. These assays have shown that plakoglobin associates most tightly with the C-domain of Dsg, to a lesser degree with that of Dsc and only weakly with the C-domain of E-cadherin. Three separate segments of plakoglobin containing various numbers of the so-called *arm* repeats exhibit distinct binding to the desmosomal cadherins comparable in strength to that of the entire molecule. The binding pattern of plakoglobin segments *in vitro* is compared with that *in vivo*. Paradoxically, *in vitro* some internal plakoglobin fragments bind even better to the C-domain of E-cadherin than the entire molecule, indicating that elements exist in native plakoglobin that interfere with the interaction of this protein with its various cadherin partners.

PLAKOGLOBIN is a common and major component of the diverse adhering junctions contributing to cell-cell adhesion and is located in their plaque anchoring cytoskeletal filaments to the plasma membrane (Cowin et al., 1986; for review see Schmidt et al., 1994). In epithelial cells, two major kinds of such plakoglobin-containing junctions are distinguished, the adherens junctions anchoring actin microfilaments and the desmosomes anchoring intermediate-sized filament (IF)¹ bundles (for reviews see Green and Jones, 1990; Schwarz et al., 1990; Garrod, 1993; Schmidt et al., 1994). As an architectural constituent of

these junctional plaques plakoglobin interacts with the cytoplasmic portions ("tails") of certain clustered cell surface glycoproteins, the cadherins, mediating calcium-dependent cell-cell adhesion (for review see Takeichi, 1990, 1991; Grunwald, 1993). In epithelial cells it is primarily E-cadherin which directs microfilament anchorage through the formation of plaque complexes containing α -catenin in combinations with β -catenin or plakoglobin (Nagafuchi and Takeichi, 1988; Ozawa et al., 1989; for review see Kemler, 1993) whereas certain cell type-specific combinations of the desmosomal cadherins, desmoglein (Dsg1)-3, and desmocollin (Dsc1)-3, define the formation of a plaque that contains plakoglobin and desmoplakin(s) and anchors IFs (Koch et al., 1992; Schäfer et al., 1994; for reviews see Buxton et al., 1993; Koch and Franke, 1994).

Furthermore, binding experiments *in vivo* and *in vitro* have shown that a very short cadherin tail region is decisively involved in plaque formation and filament anchorage. In E-cadherin, a carboxy-terminal segment, "the cate-

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1. *Abbreviations used in this paper:* aa, amino acid; Dsc, desmocollin; Dsg, desmoglein; IF, intermediate filament; RT, room temperature.

nin-binding domain," interacts with either β -catenin or plakoglobin and is crucial for microfilament anchorage (Nagafuchi and Takeichi, 1988; Ozawa et al., 1990; Stapert and Kemler, 1994; Jou et al., 1995) whereas the corresponding short segment of the C-domain of desmosomal cadherins (Koch et al., 1990), located carboxyterminally in Dsc molecules and more centrally in Dsg tails, has been shown to be essential for desmosome plaque formation and IF attachment (Trojanovsky et al., 1993, 1994a,b; Mathur et al., 1994).

The central portion of plakoglobin, comprising \sim 560 aa, is very similar in the amino acid sequence to that of β -catenin (\sim 85% identity), while the flanking amino- and carboxy-terminal regions of \sim 120 and 60 aa, respectively, are divergent. Moreover, this central portion of plakoglobin is highly conserved in evolution and defines a multigene family of related proteins identified in diverse invertebrates including flies, sea urchin, and echinoderms (Franke et al., 1989; Peifer and Wieschaus, 1990; McCrea et al., 1991; Butz et al., 1992; Fouquet et al., 1992; Peifer, 1993; Rosenthal, 1993; Peifer et al., 1993, 1994). The central domain is composed of a set of imperfect repeats of the *arm*-motif, first defined in the *Drosophila armadillo* gene (Peifer and Wieschaus, 1990), and later in other molecules with different functions and localization (Peifer et al., 1994), including the desmosomal plaque protein, plakophilin 1 ("band 6 protein;" Schäfer et al., 1993; Hatzfeld et al., 1994; Heid et al., 1994; Schmidt et al., 1994). In several proteins of this family, this *arm*-containing segment has been identified as a multifunctional module capable of binding to different cadherins, to epidermal growth factor receptors, and/or to other *arm*-repeat containing proteins, including the product of the tumor suppressor gene APC (Knudsen and Wheelock, 1992; Su et al., 1993; Hoshuetzky et al., 1994; Hülsken et al., 1994; Rubinfeld et al., 1995; for binding of tyrosine kinase substrate, p120, to E-cadherin see Shibamoto et al., 1995).

Therefore, we have examined *in vivo* and *in vitro* the function of plakoglobin in desmosome assembly and have identified molecular domains responsible for its binding to desmosomal cadherins. In these experiments, we have also observed a site-directing ("topogenic") effect of the cadherin binding of plakoglobin on the transmembranous glycoprotein, synaptophysin, and the vesicles containing it.

Materials and Methods

Plasmid Construction

To construct a plasmid encoding the synaptophysin-plakoglobin fusion protein, SyPg, the endogenous stop codon of synaptophysin in clone pSR⁵ (Leube et al., 1987) was first replaced by a 270-bp NarI-BamHI fragment obtained from the same clone by PCR using amplimers Sy-O-1 (5'-AAA GGC GCC CCG GAA AAG CAA CCA-3') and Sy-O-2 (5'-AAA GGA TCC TCA TCT GAT TGG AGA AGG AGG-3'), resulting in plasmid BISy1. The HindIII-blunt-ended BamHI fragment from this clone was then implanted 60 bp upstream of the human plakoglobin initiation codon, i.e., between the HindIII-blunt-ended NarI sites of clone HPG Ca 2.1 (Franke et al., 1989). The resulting plasmid BISyPg encodes a chimeric polypeptide consisting of the entire rat synaptophysin molecule at the amino terminus, the complete human plakoglobin molecule at the carboxy terminus, and a short 20-amino acid-long linker element between the two (Fig. 1).

Carboxy-terminal deletion mutants of BISyPg were produced either

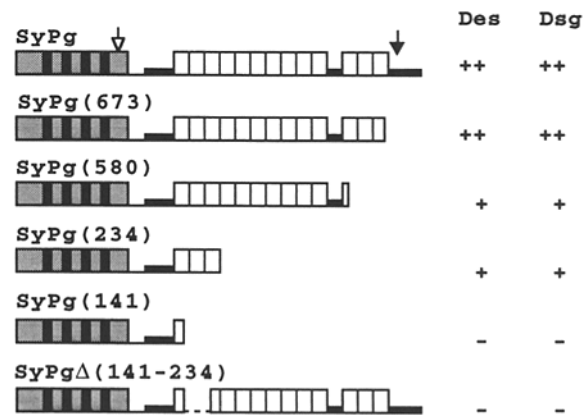


Figure 1. Schematic representation of chimeric polypeptides expressed stably in cDNA-transfected A-431 cell clones. The chimeras contained at their amino terminus full-sized rat synaptophysin (*stippled*; four transmembrane regions indicated by black boxes). This part was separated by a short linker region of 22 aa (*thin line*) from the entire 744-aa long human plakoglobin in construct SyPg. The amino- and carboxy-terminal domains of plakoglobin are demarcated by thick solid lines and the 13 internal repeats by open boxes that are interrupted by a 22-aa insertion (*central thick line*). Various parts of the plakoglobin molecule were absent in the other chimeras as indicated. The position of epitopes used for immunological detection are shown: rabbit antibodies reactive with the cytoplasmic carboxy terminus of synaptophysin (*arrow with open arrowhead*), and murine mAb Pg5.1 against plakoglobin (*arrow*). A-431 cell clones synthesizing these polypeptides were analyzed by immunofluorescence microscopy for the colocalization of chimeras at the cell surface together with desmosomal proteins in plaquelike regions (Des) and by immunoprecipitation with synaptophysin antibodies for the capacity of chimeric molecules to coprecipitate endogenous Dsg (Dsg).

with the help of conveniently located unique restriction endonuclease sites or by PCR. For the construction of BISyPg(673), primers Pg-O-6 (5'-CTT CAA CCC GGG ACC CCA-3') and Pg-O-7 (5'-TTT GGA TCC TGA AGA GGG AGT TGG TGA-3') were used in PCR from BISyPg. The SmaI-BamHI-cleaved PCR product was further cloned, using the SmaI-BamHI-digested vector BISyPg. For constructing BISyPg(580), the 280-bp-long sequence between the BglII and BamHI sites of BISyPg(673) was excised and the remaining plasmid religated. To construct BISyPg(234), a HindIII-blunt-ended SphI fragment of BISyPg was ligated into the HindIII and blunt-ended BamHI sites of Bluescript. In these cases the stop codon TGA located further downstream within the multiple cloning site of Bluescript was used. PCR-mediated site-directed mutagenesis was used to construct the plakoglobin deletion clones BISyPg(141) and BISyPg(141-234). The construction of BISyDg coding for a chimera of rat synaptophysin and bovine Dsg1 has been described previously (Trojanovsky et al., 1993).

The HindIII-XbaI inserts of all Bluescript clones were further subcloned into the eukaryotic actin promoter-driven expression vector pH β APr-1-neo, containing the neomycin resistance gene (Horst et al., 1991).

To express plakoglobin and plakoglobin mutants in *E. coli*, the QIA-expression system (Qiagen, Chatsworth, CA) was used which allows simple purification of fusion proteins containing multiple histidine residues by nickel column chromatography. First, the HindIII-SphI fragment containing the entire synaptophysin-coding region and the 5'-region of plakoglobin were excised from BISyPg(673). This fragment was then replaced by a fragment produced in a PCR from BISyPg(673) with primers Pg-O-1 (5'-AAT AAG CTT GGA TCC GAG GTG ATG AAC CTG ATG-3') containing a BamHI site in the position of the initiation codon of plakoglobin and Pg-O-2 (5'-AAA AGG CAT GCG GAC CAG AGC AGG GAT-3') containing a unique SphI site. Afterwards, either the 1,737-bp BamHI-BglII or the 2,121-bp BamHI fragment was introduced into the

appropriate sites of vector pQE-18 (Qiagen) resulting in constructs pQPg1/580 or pQPg1/673, respectively. To construct clone pQPg coding for the complete plakoglobin, a PCR fragment was amplified with Pg-O-3 (5'-TGG AGA TCT TCC GGC TCA-3') and Pg-O-4 (5'-AAA AGA TCT GGG CCA GCA TGT GGT TCT-3') and integrated into the unique BglIII site of pQPg1/580.

Further clones encoding specific plakoglobin fragments were constructed using conveniently located restriction endonuclease sites. The 440-bp BamHI-SacI fragment was excised from pQPg1/673 and inserted into the appropriate sites of vector pQE-30 (Qiagen) resulting in clone pQPg1/147; the 540-bp PstI fragment from pQPg1/673 was introduced into the PstI site of pQE-9 (Qiagen) resulting in pQPg114/292; and the 600-bp BclI fragment or 410-bp BclI-BamHI fragment was taken from pQPg1/673 and inserted into the BamHI site of pQE-32 to create clones pQPg305/505 or pQPg505/672, respectively. The 490-bp BglIII fragment was excised from pQPg and inserted into the BamHI site of pQE-32 (Qiagen) to generate plasmids pQPg580/744.

Cytoplasmic cadherin domains were cloned into *E. coli* expression vectors in the following way. The cleaved pQE-30 vector containing a blunt-ended SphI site and an opened BamHI site was ligated with the NarI-blunt-ended XbaI fragment of plasmid BICoDcI (Trojanovsky et al., 1993) in the presence of the double-stranded oligonucleotide (5'-GGA TCC GCA AAA GGC GCC-3') containing a BamHI and NarI site to produce construct pQHCDc. For constructing pQHCDg, primers Dg-O-11 (5'-AAT CCG CGG CTT GGC GAG AAA GCA TAT GCT TAT GCG-3') and Dg-O-12 (5'-AAT GAG CTC TAC CAA GAG GGA TCA GAG CTC GG-3') were used in PCR from BICoDg (Trojanovsky et al., 1993). The amplified C-subdomain-encoding Dsg1 fragment was inserted into the SacII-SacI sites of BICoDc(109) (Trojanovsky et al., 1994b) and the NarI-SacI fragment of the resulting plasmid was introduced into the appropriate sites of pQHCDc. For constructing pQHCUv, the coding sequence of the murine E-cadherin C-subdomain was first amplified from mouse liver cDNA using primers Uv-O-2 (5'-AAA CCG CGG ATC GAT GAA AAC CTC AAG GCA GCC GAC-3') and Uv-O-3 (5'-AAA AAG CTT GTC CTC GCC ACC GCC GTA-3') which were then cloned into the SacII-HindIII sites of pQHCDg.

As an alternative, glutathione fusion proteins were prepared. For constructs GSTDc and GSTDg, the BamHI-Sall fragments of pQHCDc or pQHCDg, respectively, were cloned into the appropriate sites of pGEX-4T-3 (Pharmacia, Uppsala, Sweden). The NarI-blunt-ended NotI sites of pQHCDc were then used for ligation of the NarI-blunt-ended SacI fragment of BICoDc(109). This cloning resulted in construct GSTDcΔC in which the coding sequence of the C-subdomain is lacking.

Correct construction of all recombinant plasmids was checked by restriction endonuclease mapping and/or nucleotide sequencing.

Cell Cultures, DNA Transfection, and Immunohistology

Transfection of human vulvar carcinoma-derived A-431 cells, as well as the selection and growth of stably transfected cell clones have been described (Trojanovsky et al., 1993). At least three independent positive clones for each construct, expressing approximately the same level of the recombinant product, were selected using immunofluorescence microscopy and Western blot analysis of the total lysates. Immunofluorescence microscopy on methanol/acetone-fixed cells, electron microscopy, and immunoelectron microscopy were performed as described (Leube et al., 1994). Polyclonal rabbit antibodies were used that reacted with the cytoplasmic amino terminus of synaptophysin (affinity-purified antibodies were kindly provided by Dr. Bertram Wiedenmann, University of Berlin), the cytoplasmic carboxy terminus of synaptophysin (DAKO, Hamburg, FRG; see also Leube et al., 1994 for affinity-purified antibodies), and the intracellular segment of human Dsc1a (kindly provided by Dr. Martin Demlehner et al., 1995). Murine mAbs were used that recognize bovine and human desmocolins (DC210.2.9; kindly provided Dr. Monika Schmelz, German Cancer Research Center, Heidelberg, FRG), bovine and human Dsg (Dg3.10; Schmelz et al., 1986; Koch et al., 1990), plakoglobin (PG5.1; Cowin et al., 1985, 1986), desmoplakin (DPI and DpII \pm -2.15, 2.17 and 2.19; Cowin et al., 1985), cytokeratins (lu-5; Franke et al., 1987c), synaptophysin (Sy38; Wiedenmann and Franke, 1985), the transferrin receptor (B3/25; Boehringer Mannheim), as well as α -catenin and E-cadherin (all from Transduction Laboratories, Lexington, KY).

For labeling of endosomes, cells were incubated with FITC-coupled HRP (Molecular Probes, Eugene, OR) between 3 μ g/ml and 10 mg/ml for various time periods ranging from 10 min to 2 h (cf. Leube et al., 1994).

Immunoprecipitation and Immunoblotting

Immunoprecipitation was done as described (Trojanovsky et al., 1994a). In brief, cells were lysed in hypotonic buffer (10 mM Tris-HCl, pH 7.4, 2 mM DTT, and 20 μ M 4-aminophenyl-ethansulfonyl-fluoride). After homogenization in a tight-fitting Dounce homogenizer, a postnuclear supernatant was obtained by centrifugation at 1,000 g for 5 min that was diluted with twice-concentrated immunoprecipitation buffer (100 mM Tris-HCl, pH 7.5, 2% NP-40, 300 mM NaCl, 20 μ M 4-aminophenyl-ethansulfonyl-fluoride and 4 mM EDTA). The lysate was then centrifuged at 20,000 g for 10 min and the resulting supernatant was incubated with rabbit antibodies against the cytoplasmic carboxy terminus of synaptophysin (Dako) overnight at 4°C. An incubation with 15 μ g protein A-Sepharose (Pharmacia P-L Biochemicals Inc., Milwaukee, WI) suspended in immunoprecipitation buffer followed. The beads were then washed five times with PBS and one time with PBS supplemented with 1% Triton X-100. The immunoprecipitates were analyzed by immunoblotting with different primary antibodies in conjunction with an enhanced chemiluminescence detection system (Boehringer Mannheim).

Purification of Recombinant Proteins from *E. coli*

GST fusion proteins were produced and purified basically as described by Smith and Johnson (1988). Briefly, logarithmically growing cultures of transformed *E. coli* M-15 cells were incubated with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) for 5 h at 30°C. The cells were pelleted, resuspended in 10 ml of ice-cold PBS (10 mM phosphate buffer, pH 7.4; 0.165 M NaCl) supplemented with 1 μ M PMSF. After extensive sonication, cell debris was removed by centrifugation and the supernatant was applied to glutathione-Sepharose 4B beads (Pharmacia). GST-containing fusion proteins were eluted with 10 mM glutathione in 50 mM Tris-HCl, pH 8.0.

Histidine-tagged proteins were isolated essentially as described by Qiagen. Briefly, the synthesis of the tagged proteins was induced by incubation of bacteria with 1 mM IPTG for 4 h at 37°C. The cells were harvested by centrifugation and resuspended in lysis buffer (6 M guanidine hydrochloride; 0.1 M Na-phosphate, 0.01 M Tris-HCl, pH 8.0, 1 μ M PMSF, and 10 mM DTT). The lysates were stirred for 1 h and centrifuged at 10,000 g for 15 min at 4°C. The supernatant was then applied to a Ni-NTA resin-containing column (Qiagen). Columns were washed consecutively with lysis buffer, buffer B (the same as lysis buffer, but with 8 M urea instead of guanidine hydrochloride, pH 8.0), and buffer C (the same as B, but pH 6.3). Recombinant proteins were eluted in buffer D (the same as B, but pH 5.9).

Multiwell Solid-Phase Binding Assay

Recombinant plakoglobin or plakoglobin fragments were diluted in loading buffer (20 mM Tris HCl, pH 7.8, 2 mM EDTA, 1 mM DTT) to a final concentration of 10 μ g/ml. 100 μ l of this solution were loaded in individual wells of a 96-well microtiter plate and incubated for 1 h at room temperature (RT). After removal of the solution, wells were incubated for 30 min in a 200- μ l blocking solution (0.5% bovine serum albumin in PBS, pH 7.2, 0.05% Tween 20). Different amounts of recombinant cadherin tails were diluted in blocking solution and added for 40 min at RT. After extensive washing, binding was evaluated using monoclonal antibody DC210.2.9 or anti-GST goat antibodies (Pharmacia), followed by incubation with HRP-conjugated secondary antibodies (Promega, Madison, WI, or Sigma Chem. Co., St. Louis, MO) and by ABTS immunoassay substrate incubation (Boehringer Mannheim). Reactions were monitored at 405 nm. Nonspecific binding was determined using GSTDcΔC recombinant polypeptide diluted to the same molar concentration as the other recombinant cadherin tail domains.

Overlay Binding Assays

Recombinant plakoglobin fragments (0.8 μ g per lane) were separated by SDS-PAGE and electroblotted onto nitrocellulose. Unspecific binding was blocked by treatment with 3% BSA in PBS. Membranes were then incubated with 20 μ g/ml recombinant fusion proteins GSTDg, GSTDc, GSTDcΔC, GST, or HCUv for 60 min at room temperature. After several washes with PBS/Tween 20 (0.1%), membranes were incubated with goat antibodies against GST, or, in the case of HCUv, with antibody DC210.2.9, and the immunoreaction was detected by the alkaline phosphatase system (Sigma).

Results

Synaptophysin-bound Plakoglobin Attaches Small Vesicles to Desmosomes

Using transfection with gene constructs encoding chimeric proteins of the transmembrane part of the gap junction protein, connexin32, and cytoplasmic segments of various cadherins, we have previously shown that, in living epithelial cells, binding of plakoglobin to a defined segment of the C-domain of Dsg and Dsc is necessary for the recruitment and assembly of other desmosomal plaque proteins into a plaque structure competent in anchoring IFs (Trojanovsky et al., 1993, 1994a,b). By contrast, chimeras containing the neuroendocrine vesicle protein synaptophysin and the cytoplasmic tail of the desmosomal cadherins Dsg1 or Dsc1a did not affect desmosome formation in A-431 cells as the chimeric protein accumulated in cytoplasmic vesicles, binding plakoglobin (Trojanovsky et al., 1993) but not desmoplakin (Fig. 2, *a* and *a'*). To further examine the role of plakoglobin in the formation of desmosome-equivalent structures *in vivo* we have prepared fusion proteins in which the transmembrane portion of connexin or synaptophysin was covalently combined with the entire plakoglobin polypeptide. The chimeric connexin-plakoglobin protein efficiently assembled into gap junctionlike structures at cell contact regions of stably transfected A-431 cells where it colocalized with desmoplakin, both kinds of desmosomal cadherins and IF proteins (not shown). A different distribution, however, was noted for plakoglobin covalently linked to synaptophysin in the chimeric protein SyPg (Fig. 1) produced upon transfection of A-431 cells: (*a*) in contrast to chimeras SyDg and SyDcl, only a minor proportion of SyPg was seen in the punctate pattern of cytoplasmic vesicles, as expected for synaptophysin-containing molecules (cf. Fig. 2 *a* and Leube et al., 1989, 1994) whereas most of the immunofluorescence appeared in large cytoplasmic structures and at extended cell contact regions (Fig. 2, *b–d*). (*b*) Colocalization of SyPg with Dsg was prominent at the cell surface and in the large cytoplasmic structures but was not readily detected in the few small synaptophysin-positive vesicles (Fig. 2, *b* and *b'*). (*c*) SyPg codistributed with desmoplakin at the cell contact sites but not in the cytoplasmic vesicles and aggregates (Fig. 2, *c* and *c'*). (*d*) IF bundles appeared to associate for long distances with SyPg in regions of cell contacts (Fig. 2, *d* and *d'*). (*e*) The large cytoplasmic structures could be shown upon cultivation of the transfected cells for short periods of time in the presence of FITC-conjugated HRP to be labeled by HRP and also colocalized with the transferrin receptor but not with the lysosomal markers LAMP1 or LAMP2 (data not shown).

The structures containing the chimeric protein SyPg at cell contact sites were identified by electron microscopy to represent desmosomes with an altered appearance. Such stably transfected cell clones (e.g., ASyPg6) contained numerous, relatively small desmosomal junctions with a somewhat less tightly packed plaque (compare Fig. 3 *a* with 3 *b*) associated with many small, empty-looking vesicles which often seemed to be sandwiched between the plaque and an associated IF bundle. Analysis of serial sections revealed that these structures were true vesicles of

the size expected of synaptophysin-rich cytoplasmic vesicles (Wiedenmann and Franke, 1985; Leube et al., 1994) and not just invaginations of the plasma membrane.

Immunoelectron microscopy showed that these vesicles did indeed contain plakoglobin in the molecular context with synaptophysin (Fig. 3 *c*). In addition, several small SyPg-positive vesicles were also labeled in cell-cell contact regions devoid of desmosome-like structures. Remarkably, little immunogold was detected in nonvesicular structures including the plasma membrane. These observations suggest that plakoglobin linked to synaptophysin in the SyPg chimera is recovered in small vesicles resembling presynaptic vesicles but has a strong topogenic influence as these vesicles no longer recycle deep into the cell interior but tend to remain associated with the desmosomal plaque structure. Obviously, the topogenic effect of plakoglobin overrides the normal synaptophysin effect on the behavior and distribution of such vesicles (cf. Leube et al., 1989, 1994; Cameron et al., 1991; Linstedt and Kelly, 1991).

Chimeric Protein SyPg Associates with Desmosomal Cadherins

It has been reported that soluble plakoglobin can form dimeric complexes (Kapprell et al., 1987) and that structure-bound plakoglobin can be detected in ternary complexes with cadherins, α -catenin, and p120 (Korman et al., 1989; Knudsen and Wheelock, 1992; Hülsken et al., 1994; Näthke et al., 1994; Shibamoto et al., 1995). To examine which interaction may occur between the chimeric SyPg molecules and endogenous proteins, we performed immunoprecipitation experiments, using lysates from ASyPg6 cells, synaptophysin antibodies, and immunoblot detection of the proteins bound to the chimeras. Fig. 4 shows the result of such an experiment, demonstrating SyPg as a single polypeptide of the expected molecular mass (ca. 120 kD). While the endogenous plakoglobin was abundantly present in total cell lysates (arrowhead, lane 1 of Fig. 4 *a*), no such reactivity was detected in our immunoprecipitates (lane 2 of Fig. 4 *a*). In contrast, Dsg (Fig. 4 *b*, lane 2), Dsc (Fig. 4 *c*, lane 2) and minor amounts of α -catenin (Fig. 4 *d*, lane 2) and E-cadherin (not shown) were detected in SyPg complexes.

Regions of the Central Repeat Domain Responsible for Efficient Recruitment of SyPg into Desmosomes

To identify the plakoglobin region responsible for the accumulation of SyPg-containing vesicles at desmosomes, we constructed several deletion mutants (Fig. 1). The chimera SyPg(673) lacking the carboxy-terminal plakoglobin-specific sequence had the same intracellular distribution as SyPg. The removal of a larger region encompassing the last 3 of the 13 central *arm* repeats (SyPg[580]) markedly reduced but still affected the appearance of the transgene product near desmosome-like structures and abolished the formation of the large intracellular aggregates. Here, a remarkable heterogeneity was noted: the majority of SyPg(580)-positive vesicles were rather evenly distributed along cell-cell contact regions or dispersed throughout the cytoplasm, similar to typical synaptophysin-containing vesicles of transfected cells (Fig. 5; for comparison see Leube et al., 1989, 1994) whereas another proportion of SyPg(580)

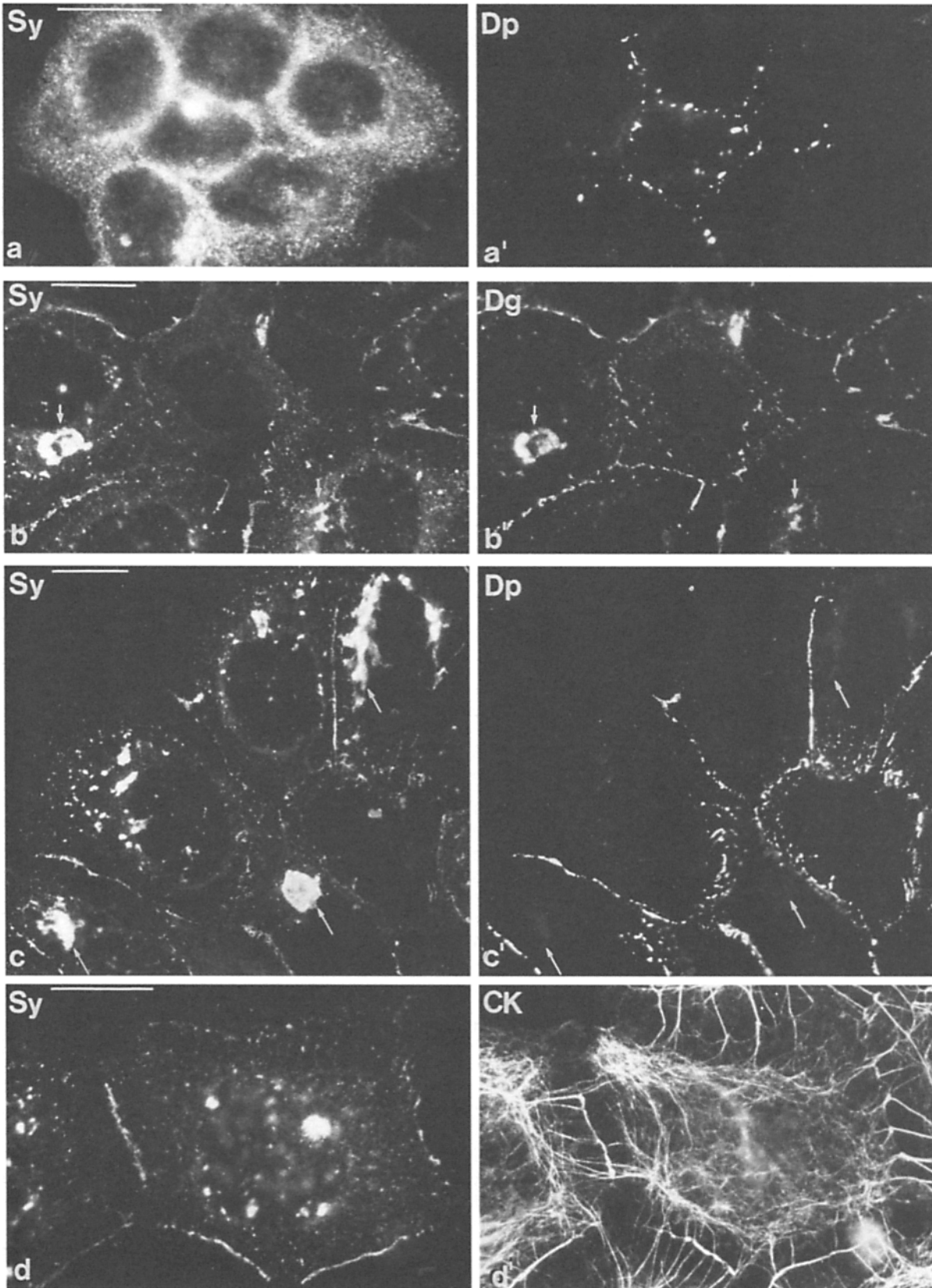


Figure 2. Double immunofluorescence microscopy detecting synaptophysin chimeras in stably transfected A-431 subclones. (*a* and *a'*) Colocalization of synaptophysin-Dsg chimera SyDg (*a*) and desmoplakin (*a'*). Note the different distribution of SyDg-containing vesicles and desmosomes. (*b–d'*) Colocalization of the synaptophysin-plakoglobin chimera SyPg (*b*, *c*, and *d*) together with desmoglein (*Dg*; *b'*), desmoplakin (*Dp*; *c'*), and cytokeratins (*CK*; *d'*) in A-431 clone ASyPg6. Note the significant codistribution in regions of cell–cell contact and the costaining of cytoplasmic structures in *b* and *b'* but not *c* and *c'* as indicated by arrows in the corresponding position and the anchorage of cytokeratin filament bundles at the cell contact sites. Bars, 20 μm .

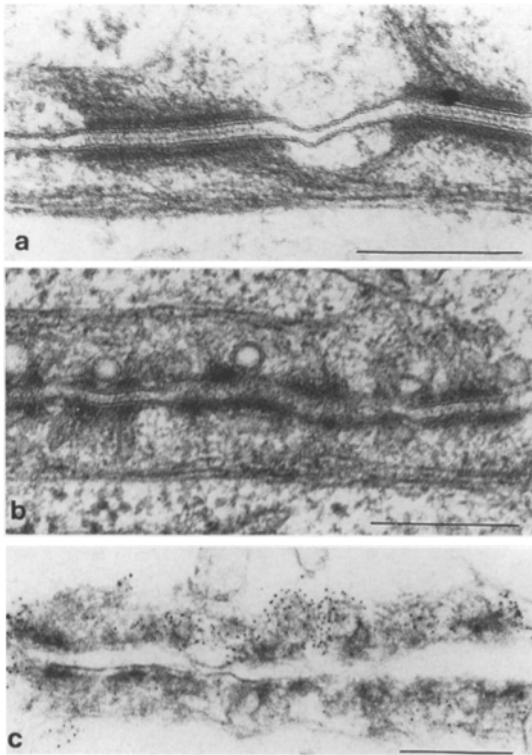


Figure 3. Altered desmosomal structures as seen in an electron microscopic comparison of A-431 clone ASyPg6 expressing chimera SyPg to nontransfected A-431 cells. (a–c) Details of cell contact regions containing desmosomes in normal A-431 cells (a) and in ASyPg6 cells (b and c). While intermediate filament bundles insert directly into the dense desmosomal plaque material (a), numerous vesicles are attached with these plaques in the ASyPg6-expressing cells (b). The SyPg polypeptide is demonstrated by the specific decoration of these vesicles by immunogold electron microscopy (c). Bars, 250 nm.

colocalized with Dsg or with desmoplakin in desmosomal structures (see arrows in Fig. 5). Even chimera SyPg(234) with a much longer truncation of the *arm*-repeat domain was still integrated to a significant proportion into cell–cell contacts, (Fig. 6, a and a') approximately with the same efficiency as SyPg(580). The reason for this heterogeneity of reaction of the same deletion construct in the same cell is not understood. The two other mutants, SyPg(141) and SyPgΔ(141–234), did not codistribute with desmosomes and were seen in a multipunctate cytoplasmic distribution indistinguishable from wild-type synaptophysin or the chimeras SyDg and SyDcI (Fig. 6, b and b').

Immunoprecipitation experiments demonstrated that SyPg(580) and SyPg(234) still bind Dsg but apparently with lower efficiency or affinity than SyPg (Fig. 7 b): the relative immunoreaction of Dsg coprecipitated was consistently below approximately a third of that seen in the SyPg coprecipitates. Deletion of the second and third *arm* repeat in SyPg(141) and SyPgΔ(141–234) completely abolished the association of these mutants with Dsg (Fig. 7). These data indicate that, at least in the SyPg context, the last three as well as the second and third *arm* repeats in plakoglobin make important contributions to the efficient association of plakoglobin-coated vesicles to desmosomes,

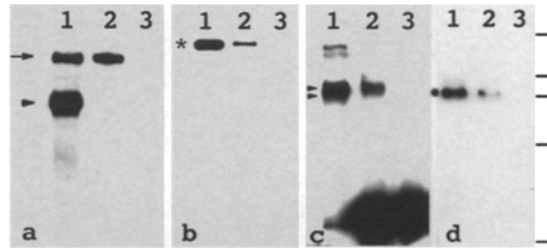


Figure 4. Immunoblot analysis of immunoprecipitates obtained from ASyPg6 cells stably expressing chimera SyPg. Total cell lysates were loaded in lanes 1, immunoprecipitates using polyclonal synaptophysin antibodies in lanes 2, and immunoprecipitates using nonimmune serum as negative control in lanes 3. Polypeptides were separated by 10% SDS-PAGE and transferred onto nitrocellulose before reaction with either plakoglobin antibody Pg5.1 (a), desmoglein antibody Dg3.10 (b), polyclonal antibodies against Dsc (c), or monoclonal α -catenin antibodies (d). Immunological reactions were detected by HRP-coupled secondary antibodies and an enhanced chemiluminescence system. The reactive polypeptides are in (a) plakoglobin (arrowhead) and chimera SyPg (arrow), in (b) Dsg (star), in (c) Dsc splice variants a and b (double arrowheads), and in (d) α -catenin (dot). Note that while the chimera and Dsg are present in the immunoprecipitate, plakoglobin is not. The nature of the weak high molecular weight bands visible in c is unknown (note the absence of these bands in the immunoprecipitates). Coelectrophoresed size markers (horizontal bars) are from top to bottom: myosin, M_r 205,000; galactosidase, M_r 116,000; phosphorylase a, M_r 97,400; BSA, M_r 67,000; ovalbumin, M_r 45,000.

and that in this specific molecular context the second and the third repeat are essential.

The Central Repeat Domain of Plakoglobin Contains Several Cadherin-binding Sites

For understanding the binding of plakoglobin in the SyPg chimeras to desmosomes and desmosomal cadherins, we examined the direct molecular interaction between these cadherins and plakoglobin *in vitro*, using recombinant polypeptides. It had been shown that the C-domain of different cadherins is needed for plakoglobin binding (Knudsen and Wheelock, 1992; Mathur et al., 1994; Troyanovsky et al., 1994a,b; Sacco et al., 1995). To optimize comparison, we therefore used recombinant cadherin fragments that differed only in the C-subdomain (Fig. 8 A): all polypeptides contained the identical intracellular anchor (IA) region of bovine Dsc1 (for domain nomenclature see Koch et al., 1990; Franke et al., 1992; Schäfer et al., 1994), carrying the epitope of mAb DC210.2.9, with either a histidine hexamer or the glutathione-S-transferase element as tags at the amino terminus to facilitate purification under denaturing (“his tag”) or nondenaturing (“GST tag”) conditions. Purified products were analyzed by SDS-PAGE (Fig. 7 b). Similarly, human plakoglobin and mutants encompassing different parts of the molecule were constructed and expressed in *E. coli* (Fig. 8, C and D).

To analyze interactions between plakoglobin and desmosomal cadherins, a solid phase assay for binding studies was developed. Plakoglobin was immobilized on a 96-well dish and incubated with increasing amounts of recombinant cadherin tails. For Scatchard analysis of binding kinetics

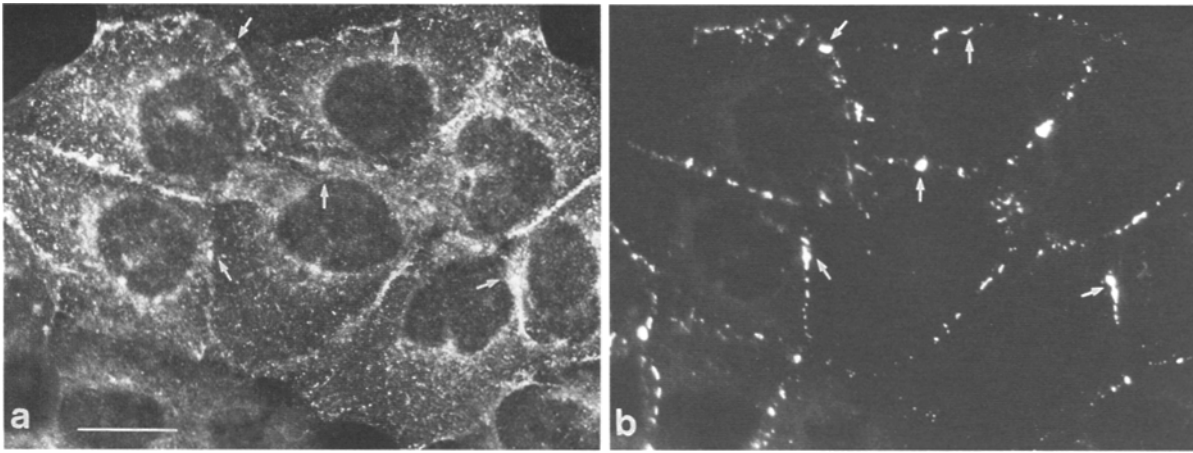


Figure 5. Double immunofluorescence microscopy detecting SyPg(580) (a) and Dsg (b) in stably transfected A-431 cells. Note that most SyPg(580)-containing vesicles are randomly distributed over the cytoplasm whereas a minor amount colocalizes with Dsg at the plasma membrane (some are denoted by arrows). Bar, 20 μm .

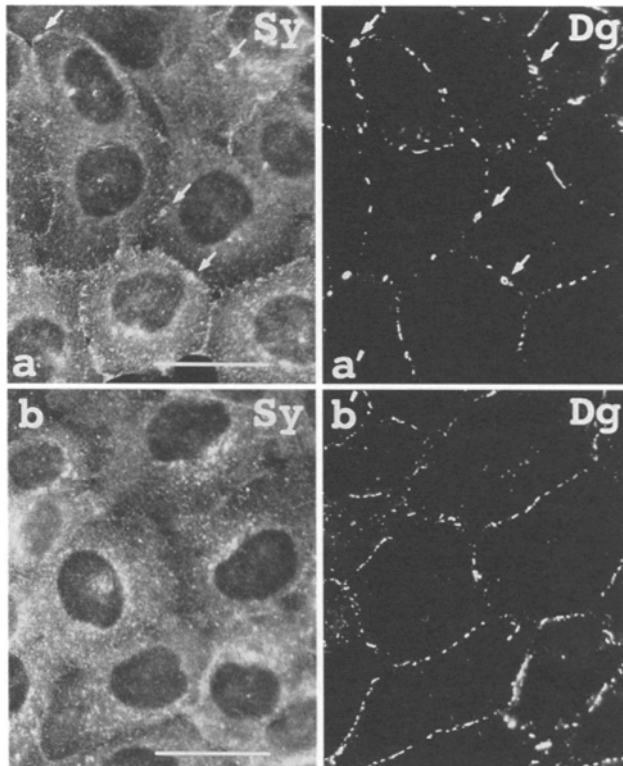


Figure 6. Double-label immunofluorescence microscopy of A-431 cells stably producing chimeras SyPg(234) (a and a') and SyPg(141) (b and b'). The left hand pictures (a and b') show the distribution of the chimeric proteins as visualized by immunostaining with rabbit synaptophysin antibodies, in comparison with the specific localization of the desmosomes as detected by mAb 3.10 against Dsg, on the right hand (a' and b'). Note that a number of the desmosomes in the cells transfected with SyPg(234), but not those in SyPg(141)-transfected cells contain detectable chimeric protein (four examples of sites of colocalization are denoted by arrows). Bar, 40 μm .

these tails were labeled with ^{125}I . This assay demonstrated that the recombinant proteins containing the C-subdomains of Dsg1 (HCDg) and of Dsc1a (HCDc) bind to plakoglobin with K_d values of 1.5×10^{-7} and 4.5×10^{-7} , respectively. The fusion protein GSTDc, containing a GST tag instead of the his tag (Fig. 8 A), binds to plakoglobin with similar affinity as HCDc. By contrast, two tested polypeptides lacking the C-subdomain (GSTDc ΔC or GST alone) failed to bind plakoglobin in this assay. These observations provide compelling proof that the C-subdomains of desmosomal cadherins are essential for the direct interaction with plakoglobin in vitro and confirm our previous in vivo results (Trojanovsky et al., 1994a,b).

Alternatively, to study the specific binding of HCDg, HCDc, and HCUv (HCUv contains the C-subdomain of

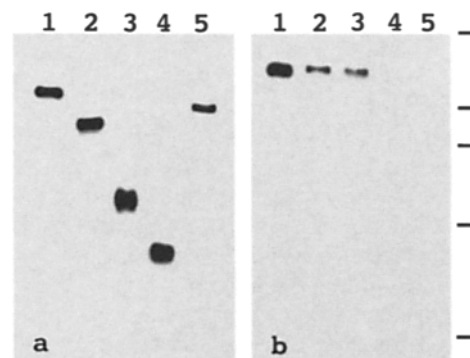


Figure 7. Immunoblot analysis of immunoprecipitates obtained with the help of polyclonal synaptophysin antibodies from A-431 cells stably expressing SyPg (lanes 1), SyPg(580) (lanes 2), SyPg(234) (lanes 3), SyPg(141) (lanes 4), and SyPg Δ (141-234) (lanes 5). SyPg-type chimeras were detected with monoclonal synaptophysin antibody SY38 (a) and Dsg with antibody Dg3.10 (b). Note that reaction in lanes 2 and 3 of b is positive but that the relative amount of Dsg is significantly reduced in SyPg(580) and SyPg(234) precipitates and undetectable in SyPg(141) and SyPg Δ (141-234) precipitates. Positions of coelectrophoresed size markers are shown by horizontal bars (from top to bottom M_r , 205,000; 116,000; 97,400; 67,000; 45,000; cf. Fig. 4).

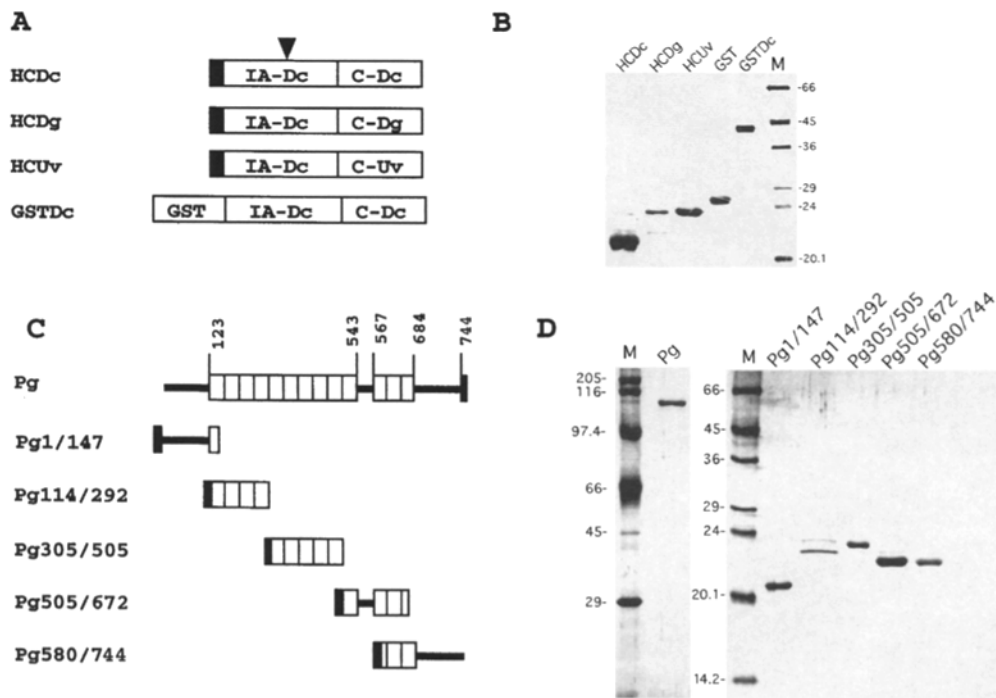


Figure 8. Schematic representation and SDS-PAGE of recombinant molecules expressed in *E. coli*. (A) Scheme of cadherin tail constructs. The polypeptides were expressed as fusion proteins containing either six histidine residues (black box) or glutathione (GST) at the amino terminus to be used for purification. Each construct contained the region coding for the intracellular anchor subdomain of bovine Dsc1 (IA-Dc) followed by C-domains from either bovine Dsc1 (C-Dc) in HCDc and GSTDc, bovine Dsg1 (C-Dg) in HCDg and murine E-cadherin (C-Uv) in HCUv. The position of the epitope for mAb DC210.2.9 used for immunological detection of recombinant proteins in binding assays is indicated by an arrowhead. (B) Coomassie

blue-stained polypeptides seen after purification of recombinant proteins from *E. coli* using either Ni-NTA-agarose for HCDc, HCDg, and HCUv or with glutathione-agarose for GSTDc. In the lane labeled GST only glutathione-S-transferase was loaded. Polypeptides were separated by 15% SDS-PAGE. Positions and approximate M_r of coelectrophoresed size markers in lane M are indicated. (C) Scheme of plakoglobin and plakoglobin mutants expressed as fusion proteins in *E. coli*. The polyhistidine tag located either at the amino or carboxy terminus is shown as a black box, amino- and carboxy-terminal domains as thick lines and the central repeats as open boxes interrupted by a short insertion (thick line). The numbers in parentheses of the construct names refer to the first and last amino acid residues in the respective plakoglobin mutants. (D) Detection of Ni-NTA-purified plakoglobin and plakoglobin fragments after SDS-PAGE (10% in first two lanes; 15% in last six lanes) by Coomassie blue staining. Size and position of coelectrophoresed MW markers in lanes M are shown.

E-cadherin) to plakoglobin or fragments thereof an ELISA assay with mAb DC210.2.9 was used. In these experiments, preabsorbed plakoglobin was incubated with fixed amounts (100 ng/ml, i.e., 0.5×10^{-7} M) of different recombinant cadherin tails. Again, the strongest binding to intact plakoglobin was observed for HCDg. Plakoglobin binding of HCDc and HCUv was two- and fivefold weaker, respectively (Fig. 9).

To find out which part(s) of the plakoglobin molecule participate(s) in the interaction with the cadherin tails, different segments of plakoglobin were tested for binding activity in the same solid phase assay. The results (Fig. 9) show that neither the amino- nor the carboxy-terminal domains (mutants Pg1/147 and Pg580/744) contributed significantly to this binding activity while three nonoverlapping fragments of the central repeat domain (mutants Pg114/292, Pg305/505, and Pg505/672) bound strongly to HCDg with an affinity only slightly less than that of intact plakoglobin. This indicates that the central domain of plakoglobin contains at least three independent binding sites for cadherins. Competition experiments showed that the binding of HCDg to the entire plakoglobin or plakoglobin fragments Pg114/292, Pg305/505, and Pg505/672 could be competed equally well by Pg505/672 (Fig. 10). This suggests that each plakoglobin fragment recognizes the same or overlapping sequences in the C-domain of Dsg.

Such binding experiments, however, also revealed that these central segments differed in their binding specific-

ties. Plakoglobin fragments, Pg305/505 and Pg505/672, showed remarkably strong binding to the E-cadherin probe HCUv, similar to that of the desmoglein probe tested in parallel, whereas fragment Pg114/292 preferentially bound the desmoglein probe HCDg as did the intact plakoglobin. The low binding of the intact plakoglobin molecule to E-cadherin, probed by HCUv (Fig. 9), might be explained by the existence of interfering sites in the native plakoglobin molecule.

As an alternative approach, the molecular interaction between wild-type and mutant plakoglobin with desmosomal cadherins and E-cadherin was tested in overlay assays. Fig. 11 shows a representative experiment in which selected plakoglobin fragments were nitrocellulose-bound and incubated with proteins GSTDg, GSTDc, or HCUv, and binding was detected by antibodies reactive with GST or DC210. The control proteins GSTDcΔC and GST did not bind in this assay to any plakoglobin fragment (not shown). The results of these experiments are in good agreement with those obtained in the solid phase binding assays, demonstrating that fragments Pg505/672 and Pg305/505 bind equally well with all three tested C-domains. In addition, GSTDg binds to Pg114/292 while GSTDc and HCUv does not.

Discussion

Plakoglobin is a structural and regulatory constituent of all

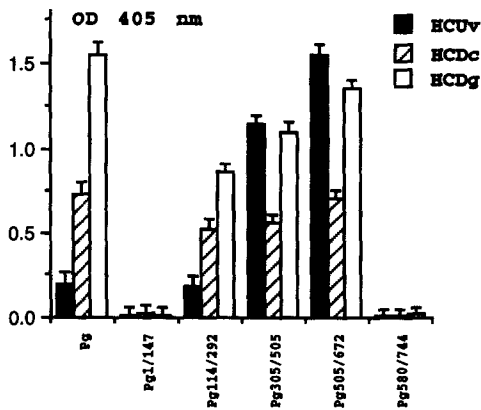


Figure 9. Histogram depicting the results of a solid-phase binding assay measuring the interactions of recombinant plakoglobin and plakoglobin fragments with different cadherin C-domains. Purified recombinant cadherin tail fragments (Fig. 8, *a* and *b*) were incubated in 96-well dishes at 100 ng/ml (0.5×10^{-7} M) in 100 μ l with plakoglobin or plakoglobin mutants that had been adsorbed to the plastic surface. Specific binding of recombinant cadherin tails was evaluated with anti-desmocollin antibody DC210.2.9, followed by HRP-coupled secondary antibodies and detection with the help of the ABTS immunoassay substratum. Reactions were monitored at 405 nm. The experiments were carried out in triplicate and one of ten independent experiments is shown. Vertical bars indicate deviations.

known plaque-bearing cell-cell junctions, the only protein common to desmosomes and the other diverse kinds of adhering junctions (Cowin et al., 1986; Franke et al., 1987*a,b*; Rose et al., 1995; for review see Schmidt et al., 1994). From the various patterns of colocalization of plakoglobin with desmosomal cadherins as well as with different "classical" cadherins such as E-, N-, VE-, and M-cadherin it appears that this protein contributes to plaque assembly and maintenance by interaction with the cadherin(s) occurring in the specific junctional structures. Because of this general importance of plakoglobin, we have extended our

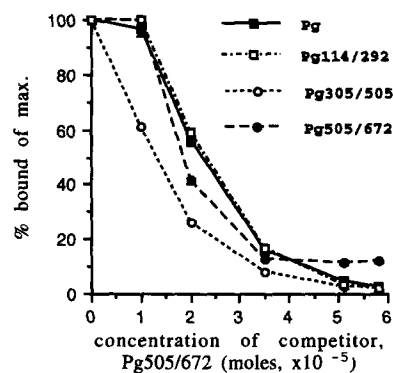


Figure 10. Competition of the binding of HCDg to plakoglobin or plakoglobin fragments by fragment Pg505/672. Wells of a multiwell dish were first coated with either plakoglobin (filled boxes), Pgl14/292 (open boxes), Pg305/505 (open circles), or Pg505/672 (filled circles) and were then incubated with HCDg (0.5×10^{-7} M) in the presence of increasing amounts of Pg505/672. The binding observed in the absence of competitor Pg505/672 is designated 100%. The results shown are the mean of duplicate determinations in each instance.

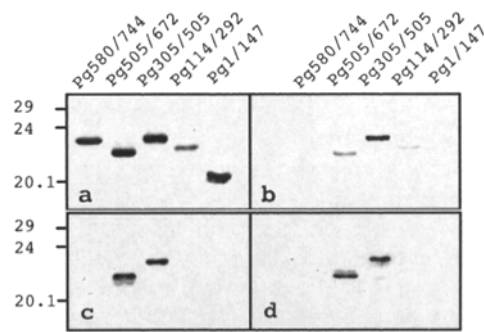


Figure 11. Blot overlay binding assay to detect the interaction of the recombinant plakoglobin fragments Pg580/744, Pg505/672, Pg305/505, Pgl14/292, and Pgl1/147 with different cadherins. The plakoglobin fragments were separated by 15% SDS-PAGE and were either stained with Coomassie blue (*a*) or transferred to nitrocellulose membranes by electroblotting. Blots were then incubated with GSTDg (*b*), GSTDc (*c*), or HCUv (*d*), and binding was detected by reaction with corresponding antibodies and visualization with the alkaline phosphatase system. Positions and sizes of coelectrophoresed reference proteins are indicated.

studies of its interaction with the desmosomal cadherins, desmoglein, and desmocollin (Trojanovsky et al., 1993, 1994*a,b*), by binding experiments probing for potential complex partners of plakoglobin *in vivo* and *in vitro* (see also Kowalczyk et al., 1994; Mathur et al., 1994).

The most remarkable result of our transfection experiments using chimeric proteins linking plakoglobin to the vesicle-constituent transmembrane glycoprotein, synaptophysin, is the finding that the binding of plakoglobin to the desmosomal cadherins, Dsg and Dsc, does not depend on the soluble state known to exist in most cells (Kapprell et al., 1987; Hinck et al., 1994; Näthke et al., 1994). Plakoglobin binds to Dsc and Dsg avidly and efficiently, even when covalently integrated into a large structure, i.e., a synaptophysin-containing vesicle. This strong and stable association can even give rise to the formation of a new structural arrangement, i.e., desmosomal arrays with numerous plaque-attached vesicles. This suggests that one of the architectural functions of plakoglobin is based on its ability to attach itself as well as other plakoglobin-binding molecules to cadherins, and thus to special sites of the plasma membrane. One known such partner of plakoglobin is α -catenin which can form a plakoglobin complex contributing to the adhering junction plaque anchoring actin microfilaments (e.g., Hinck et al., 1994; Hülsken et al., 1994; Näthke et al., 1994).

Truncation of the *arm*-repeat region of plakoglobin reduces both the binding of the SyPg-type chimeras to Dsg and the association of SyPg vesicles with desmosomes. The complete loss of Dsg binding in SyPg(141) or SyPg Δ (141-234), in comparison with SyPg(234), suggests that the second and third *arm* repeats are important for the plakoglobin-Dsg interaction *in vivo*. The reduced but still significant Dsg binding of SyPg(580) and SyPg(234) may indicate either that these deletions strongly change the conformation of the Dsg-binding domain of plakoglobin or that a segment in the missing region is normally involved in stabilizing the Dsg/plakoglobin complex or in targeting it to cell junctional location. In support of the last assumption we found that chimera CoPg(580) containing the sequences of

connexin32 and the amino-terminal 580 aa of plakoglobin efficiently forms gap junction-like structures colocalizing with desmoplakin and desmoglein (Trojanovsky, S.M., unpublished results). The marked topogenic differences between CoPg(580) and SyPg(580) seem to be caused by the dominant assembly of connexin32 into connexons stabilized in cell-cell contacts.

Our experiments on plakoglobin binding in vitro support our results obtained with chimeric molecules of transfected cells, also pointing to the important role of specific segments of the arm-repeat region in SyPg/cadherin interaction. In these experiments, the direct binding of plakoglobin to the C-domains of Dsg1, Dscl1a, and E-cadherin was compared (binding of plakoglobin to this domain has also been reported by Aberle et al., 1994; Mathur et al., 1994; Trojanovsky et al., 1994a,b; Jou et al., 1995). Despite the high degree of amino acid sequence conservation in the C-domain of these three cadherins, significantly different plakoglobin-binding affinities can be noted, suggesting that in the living cell plakoglobin is attracted by and bound to different cadherins and hence different junctions with different kinetics.

A further remarkable result of our characterizations of possible cadherin-binding sites in the plakoglobin molecule is the existence of multiple, nonoverlapping segments in the central arm-repeat domain, each with considerable binding which, in several instances, is not weaker than that of the entire molecule. Furthermore, three segments of the central repeat domain have been shown to exhibit different cadherin-binding specificities: for example, the NH₂-terminal segment Pg114/292 clearly shows the relatively highest binding to Dsg whereas other segments (Pg305/505 and Pg505/672) bind E-cadherins equally well or even better. Yet, in vitro all three segments compete efficiently with each other for cadherin binding. Remarkably, similar intensities of Dsg binding comparable to the entire plakoglobin molecule were observed in various segments along the repeat domain (see Fig. 9).

Whether these multiple cadherin-binding sites noted in vitro indeed interact with cadherins in vivo or whether their binding may be influenced by abnormal conformation(s) remains still to be examined. It is notable, however, that in vivo the same or a similar truncation mutant of plakoglobin binds differently to classical cadherins and Dsg. For example, a plakoglobin mutant containing only the 375-amino-terminal amino acids (Sacco et al., 1995) or our mutant SyPg(234) (Trojanovsky, S.M., unpublished results) are not able to associate with N-cadherin or E-cadherin, respectively. This may suggest that in vivo different sites of plakoglobin are involved in binding to the specific cadherins and that the availability of a specific binding segment may be regulated by changes of conformation or interaction with other proteins.

We thank Christine Grund and Jutta Müller-Osterholt for expert technical assistance as well as Irmgard Weirich (Heidelberg, FRG) for patient typing the manuscript. We would also like to thank Dr. Gregory I. Goldberg (St. Louis, MO) for valuable discussion.

The work has been supported by grants from the Dermatology Foundation and the Deutsche Forschungsgemeinschaft.

Received for publication 26 July 1995 and in revised form 6 February 1996.

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