Tyrosine-phosphorylated Plakoglobin Is Associated with Desmogleins but Not Desmoplakin after Epidermal Growth Factor Receptor Activation*

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Tyrosine phosphorylation of junctional components has been proposed as a mechanism for modulating cell-cell adhesion. Although a correlation exists between the tyrosine phosphorylation of the adherens junction protein β-catenin and loss of classical cadherin-mediated adhesion, the effects of tyrosine phosphorylation on the function of the adherens junction and desmosome-associated protein plakoglobin is unknown. In the present study, we investigated the effects of epidermal growth factor receptor (EGFR) tyrosine kinase activation on the subcellular distribution of plakoglobin and its association with its junctional binding partners. Long term epidermal growth factor (EGF) treatment of A431 cells revealed a modest decrease in the cytoskeleton-associated pool of plakoglobin (Pg) and a corresponding increase in the cytosolic pool of Pg. After short term EGF treatment, plakoglobin was rapidly phosphorylated, and tyrosine-phosphorylated Pg was distributed predominantly in a membrane-associated Triton X-100-soluble pool, along with a co-precipitating high molecular weight tyrosine-phosphorylated protein identified as desmoglein 2. Analysis of deletion and point mutants defined the primary EGFR-dependent targets as one or more of three C-terminal tyrosine residues. Whereas phosphorylated Pg remained associated with the desmoglein tail after both short and long term EGFR activation, no phosphorylated Pg was found associated with the N-terminal Pg-binding domain (DPNTP) of the intermediate filament-associated protein, desmoplakin. Together these results are consistent with the possibility that EGFR-dependent tyrosine phosphorylation of Pg may modulate cell-cell adhesion by compromising the link between desmosomal cadherins and the intermediate filament cytoskeleton.

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Tyrosine phosphorylation of junctional components has been proposed as a mechanism for modulating cell-cell adhesion. Although a correlation exists between the tyrosine phosphorylation of the adherens junction protein β-catenin and loss of classical cadherin-mediated adhesion, the effects of tyrosine phosphorylation on the function of the adherens junction and desmosome-associated protein plakoglobin is unknown. In the present study, we investigated the effects of epidermal growth factor receptor (EGFR) tyrosine kinase activation on the subcellular distribution of plakoglobin and its association with its junctional binding partners. Long term epidermal growth factor (EGF) treatment of A431 cells revealed a modest decrease in the cytoskeleton-associated pool of plakoglobin (Pg) and a corresponding increase in the cytosolic pool of Pg. After short term EGF treatment, plakoglobin was rapidly phosphorylated, and tyrosine-phosphorylated Pg was distributed predominantly in a membrane-associated Triton X-100-soluble pool, along with a co-precipitating high molecular weight tyrosine-phosphorylated protein identified as desmoglein 2. Analysis of deletion and point mutants defined the primary EGFR-dependent targets as one or more of three C-terminal tyrosine residues. Whereas phosphorylated Pg remained associated with the desmoglein tail after both short and long term EGFR activation, no phosphorylated Pg was found associated with the N-terminal Pg-binding domain (DPNTP) of the intermediate filament-associated protein, desmoplakin. Together these results are consistent with the possibility that EGFR-dependent tyrosine phosphorylation of Pg may modulate cell-cell adhesion by compromising the link between desmosomal cadherins and the intermediate filament cytoskeleton.

The abbreviations used are: Pg, plakoglobin; EGF, epidermal growth factor; EGFR, EGF receptor; DP, desmoplakin; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; PAGE, polyacrylamide gel electrophoresis; HRP, horseradish peroxidase; Dsg2, desmoglein 2; Ab, antibody.
ylation of β-catenin and Pg may lead to a decreased association of these proteins with the actin cytoskeleton. Through its ability to bind to either classic or desmosomal cadherin tails, Pg is a component of both desmosomes and adherens junctions and serves as a link between members of the cadherin family of proteins and cytoskeletal associated proteins (3, 17, 18). Therefore, tyrosine phosphorylation of Pg may affect both classical and desmosomal cadherin-based adhesion. Although there is evidence suggesting that tyrosine phosphorylation of β-catenin may lead to a decreased association with the cytoskeleton, less is known about the effects of tyrosine phosphorylation on Pg and its association with cytoskeletal components. We sought to examine the effect of EGF treatment on Pg with the ultimate goal of understanding the possible contribution of Pg phosphorylation on the regulation of junctional interactions during EGF-triggered cell migration. We investigated the effect of long term and short term EGF treatment on the subcellular distribution of Pg and Pg truncation mutants using biochemical approaches in epithelial cells that assemble both desmosomes and adherens junctions. We detected modest shifts in the subcellular distribution of Pg during cell migration occurring after 24 h of EGF treatment. We confirmed that endogenous Pg in A431 cells was rapidly phosphorylated on one or more of three C-terminal tyrosine residues in response to EGF and was distributed exclusively in the membrane-soluble, non-junctional pool of proteins. Furthermore, the interaction between Pg and the desmoglein tail was maintained, no interaction was detected between phosphorylated Pg and the N-terminal domain of desmoplakin (DP), which provides a link between the desmosomal plaque and the intermediate filament cytoskeleton (19, 20). Collectively, these results are consistent with the idea that tyrosine phosphorylation of the Pg C terminus affects the ability of Pg to assemble into functional junctions by compromising anchorage of the intermediate filament cytoskeleton through DP, thus contributing to the remodeling of epithelia in response to growth factors during wound healing and invasion.

EXPERIMENTAL PROCEDURES

Generation of Plakoglobin cDNA Constructs—Full-length human plakoglobin cDNA was isolated and subcloned into the mammalian expression vector LK 444 under the control of the human β-actin promoter (21). N- and C-terminal deletions of Pg were generated and subcloned into the LK 444 vector as described previously (22). Pg point mutants were generated using an overlap extension strategy. The mutants were subcloned into the LK 444 vector as described previously (22). Pg point mutants were generated using an overlap extension strategy. The mutants were subcloned into the LK 444 vector as described previously (22).

For immunoblotting, the following antibodies were used: a mouse monoclonal antibody against the c-Myc epoxide tag, 9E10.2 (24); a mouse monoclonal antibody 11E4, directed against the N terminus of plakoglobin (21); a rabbit polyclonal antibody directed against the C terminus of plakoglobin (25); and a rabbit polyclonal antibody NW161, directed against the N-terminal domain of desmoplakin (19). 11E4 was provided by Dr. M. Wheelock (University of Toledo), and the Pg C-terminal antibody was provided by Dr. J. Papkoff (Valentis Corporation, Burlingame, CA). The mouse monoclonal β-catenin antibody used for immunoprecipitations was purchased from Transduction Laboratories, Lexington, KY. The 5H10 mouse monoclonal β-catenin antibody and the desmoglein 2 monoclonal antibody 6D8 were kindly gifts from Dr. M. Wheelock. The 795 rabbit polyclonal anti-E-cadherin antibody was a kind gift from Dr. R. Marsh. The 1407 polyclonal antibody was raised in chickens using 400 μg of recombinant full-length Myc-tagged human Pg to immunize each animal (immunizations performed by Aves Labs, Tigard, OR). The anti-phosphotyrosine mouse monoclonal antibody 4G10 and the anti-Shc rabbit polyclonal antibody were purchased from Upstate Biotechnology Inc., Lake Placid, NY. The anti-phosphotyrosine mouse monoclonal antibody 4G10 and the anti-Shc rabbit polyclonal antibody were purchased from Upstate Biotechnology Inc., Lake Placid, NY.

Growth Factor Treatment—COS-7 cells, 24 h post-transfection or on glass coverslips in 35-mm culture dishes were rinsed with complete PBS then incubated 12–24 h in serum-free DMEM containing 10% bovine serum albumin, 100 units/ml penicillin, and 100 μg/ml streptomycin. A431 cells lines expressing N- and C-terminal truncations of Pg were described previously (22) and maintained in media containing 350 μg/ml (active concentration) G418. A431 clones PgΔN(7) and PgΔC (10) were used for the analyses reported here; however, similar results were seen for multiple clones expressing PgΔN or PgΔC. An A431 stable cell line, tetracycline-inducible for the expression of DP-NTP, was established as described (23). DP-NTP expression was induced using 2 μg/ml doxycycline. For transient transfections, COS-7 cells were cultured in DMEM containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin and transfected using the calcium phosphate precipitation method described previously (21).

Antibodies—The following antibodies used in this study were previously described: a mouse monoclonal antibody against the c-Myc epitope tag, 9E10.2 (24); a mouse monoclonal antibody 11E4, directed against the N terminus of plakoglobin (21); a rabbit polyclonal antibody directed against the C terminus of plakoglobin (25); and a rabbit polyclonal antibody NW161, directed against the N-terminal domain of desmoplakin (19). 11E4 was provided by Dr. M. Wheelock (University of Toledo), and the Pg C-terminal antibody was provided by Dr. J. Papkoff (Valentis Corporation, Burlingame, CA). The mouse monoclonal β-catenin antibody used for immunoprecipitations was purchased from Transduction Laboratories, Lexington, KY. The 5H10 mouse monoclonal β-catenin antibody and the desmoglein 2 monoclonal antibody 6D8 were kindly gifts from Dr. M. Wheelock. The 795 rabbit polyclonal anti-E-cadherin antibody was a kind gift from Dr. R. Marsh. The 1407 polyclonal antibody was raised in chickens using 400 μg of recombinant full-length Myc-tagged human Pg to immunize each animal (immunizations performed by Aves Labs, Tigard, OR). The anti-phosphotyrosine mouse monoclonal antibody 4G10 and the anti-Shc rabbit polyclonal antibody were purchased from Upstate Biotechnology Inc., Lake Placid, NY. The anti-phosphotyrosine mouse monoclonal antibody 4G10 and the anti-Shc rabbit polyclonal antibody were purchased from Upstate Biotechnology Inc., Lake Placid, NY.
Cells—Cells were grown to 80% confluence on 60-mm culture dishes, treated with EGF, rinsed in complete PBS, and subjected to sequential detergent extraction as described previously (22). For subsequent immunoprecipitation, 1 ml of detergent buffer was used for extraction (22). Triton X-100-insoluble proteins were solubilized in 100 μl of solubilization buffer (10 mM Tris, pH 7.5, 1% SDS, 5 mM EDTA, 2 mM EGTA, 0.1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride), heated at 95°C for 10 min to aid in solubilization, diluted with 900 μl of dilution buffer (15 mM Tris, pH 7.5, 5 mM EDTA, 2 mM EGTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 120 mM NaCl, 25 mM RCI, 0.1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride), and centrifuged at ~14,000 × g for 30 min at 4°C prior to immunoprecipitation. Immunoprecipitations were conducted using the appropriate antibody as described previously (22). Samples were subjected to SDS-PAGE on 7.5% gels and subsequent immunoblot analysis.

**RESULTS**

**Long Term EGF Treatment Results in Shifts in the Subcellular Distribution of Plakoglobin**—In response to treatment with the pro-migratory growth factors EGF or hepatocyte growth factor, epithelial cells such as HT29 or A431 scatter and lose their intercellular connections (27, 28). Phase contrast microscopy confirmed the effect of long term EGF treatment on A431 cells (Fig. 1A). In the absence of EGF, A431 cells remained in compact colonies (Fig. 1A, a). After 24 h of EGF treatment the cells acquired a more motile, fibroblastic morphology (Fig. 1A, b). In order to determine the subcellular distribution of the adherens junction and desmosome component Pg after prolonged EGF treatment, immunofluorescence using a monoclonal antibody against Pg was performed (Fig. 1A, c and d). After 24 h of EGF treatment, the cell-cell border staining typical of Pg (Fig. 1A, c) was shifted to a more cytoplasmic localization, although some staining at cell-cell borders could still be observed (Fig. 1A, d). This observation was further confirmed by subjecting A431 cells to sequential detergent extraction to release cytosolic (saponin-soluble) proteins, membrane-bound (Triton-soluble) proteins, and (Triton-insoluble) presumably junction or cytoskeleton-associated proteins after 0, 12, or 24 h of EGF treatment. Western blot analysis of the different pools revealed a modest, reproducible increase in the cytosolic pool and a concomitant decrease in the detergent-insoluble pool of Pg (Fig. 1, B and C). The Western blot shown in Fig. 1B is representative of five different experiments, and results of ECL detection were confirmed by reprobing with an 125I-conjugated secondary antibody (data not shown). Data from three experiments were quantified using the Molecular Analyst software and plotted as percentage of total plakoglobin (Fig. 1C, upper graph). Results were also expressed as the difference between the percentage of total plakoglobin present in the Triton-insoluble pool and the cytosolic pool. This difference in the percentage of total Pg significantly decreases (p < 0.05) over the time course of EGF treatment (Fig. 1C, lower graph), thus illustrating the shift in Pg solubility upon long term EGF treatment. In addition to these alterations seen when normalizing to total Pg, a decrease in total Pg levels was frequently observed, possibly due to increased turnover of protein in the soluble, non-junctional cell compartment (not shown). While this decrease was taken into account, alterations in each subcellular fraction are even more pronounced.

**Plakoglobin Is Rapidly Phosphorylated in Response to EGF and Tyrosine-phosphorylated Plakoglobin Is Predominantly in a Triton-soluble Pool**—Although Pg has been shown to be tyrosine-phosphorylated in response to growth factors such as EGF and hepatocyte growth factor/Scatter Factor (13, 27), the effect of tyrosine phosphorylation on Pg subcellular distribution and interaction with junctional binding partners is unknown. To begin to address this question, we analyzed Pg phosphorylation status and subcellular distribution following short term EGF treatment. Different Pg constructs represented in Fig. 2 were established for these studies.

First, A431 cells were treated with EGF for 1, 2, and 5 min and subjected to a sequential detergent extraction. By using the Pg-specific antibody 11E4, endogenous Pg was immunoprecipitated from each cell fraction. The subcellular distribution of Pg in the absence of EGF was similar to its previously described (22) distribution under normal growth conditions, that is a small proportion of Pg was cytosolic but the majority of the protein was distributed between the Triton-soluble and Triton-insoluble pools (Fig. 3B, EGF 0 min). Treatment of A431 cells with EGF for 2 or 5 min did not detectably alter the subcellular distribution of total Pg (Fig. 3B, EGF 2 and 5 min). After EGF treatment, Pg was rapidly phosphorylated on tyrosine residues (Fig. 3A), consistent with previous reports (13). Although Pg protein was detected in all three subcellular pools using the Pg-specific antibody, tyrosine-phosphorylated Pg was found predominantly in the membrane-associated Triton-soluble pool of proteins and not in the Triton-insoluble, junction-associated pool (Fig. 3A).

**The Plakoglobin C Terminus Is Required for EGF-induced Tyrosine Phosphorylation—β-Catenin has been shown to be phosphorylated directly by the EGF receptor in vitro (13). Interestingly, the armadillo repeats of β-catenin were not phosphorylated, suggesting that tyrosine residues in the N and/or C termini of β-catenin were targets for EGF receptor phosphorylation. Plakoglobin, which has high structural identity to β-catenin (18), has a total of 22 tyrosine residues, 5 in the N terminus, 11 in the armadillo repeats, and 6 in the C terminus. To determine whether the Pg end domains are required for EGF-dependent tyrosine phosphorylation in vivo, A-431.PgΔC and A431.PgΔN cells lines stably expressing C- or N-terminal Pg truncations were established and subjected to EGF stimulation and sequential detergent extraction as above for endogenous Pg. The PgΔN polypeptide lacks the 5 tyrosine residues in the N terminus, whereas the PgΔC polypeptide lacks the 6 tyrosine residues in the C terminus and 1 tyrosine residue from the 13th armadillo repeat (Fig. 2).

By using a Pg antibody that recognizes both endogenous and ectopic Pg, endogenous Pg and PgΔC were immunoprecipitated from each subcellular fraction. PgΔC was detected in all three pools with the majority of PgΔC in the Triton-soluble and Triton-insoluble pools (Fig. 3D). Similar to Pg from control cells, EGF treatment did not detectably alter the subcellular distribution of the Triton-insoluble or-soluble pools; however, a small increase in the cytosolic pool of the PgΔC deletion was observed. After EGF treatment tyrosine-phosphorylated endogenous Pg was detected (Fig. 3C, asterisk), but phosphorylation of PgΔC was not detectable (Fig. 3C, arrowhead), suggesting that the Pg C terminus is required for EGF-induced tyrosine phosphorylation (Fig. 3C and Fig. 5A). A small amount of tyrosine-phosphorylated endogenous Pg was present in the cytosolic pool, consistent with its previously reported association with PgΔC (22).

In the absence of growth factor, the majority of PgΔN was distributed between the cytosolic and Triton-soluble pools, and only a small proportion was detected in the Triton-insoluble pool (Fig. 3F, EGF 0 min). Interestingly, a transient decrease of ~4-fold was observed in the Triton-insoluble pool of PgΔN after 2 min of EGF treatment in three independent experiments, reflecting an increase in the detergent solubility of PgΔN. Similar to endogenous Pg from control cells and in contrast to
PgΔC, PgΔN was rapidly phosphorylated on tyrosine residues in response to EGF (Fig. 3E, EGF 2 and 5 min). However, unlike tyrosine-phosphorylated Pg from control cells, tyrosine-phosphorylated PgΔN was detected in both the cytosolic and Triton-soluble pools, with the majority of the phosphorylated protein found in the cytosolic pool. Tyrosine-phosphorylated endogenous Pg from A431.PgΔN cells was detected mainly in the Triton-soluble pool and the cytosolic pool of proteins from time 0 to 24 h is statistically significant as assessed by a two-tailed t test (p = 0.03).

Tyrosine-phosphorylated Desmoglein 2 Is in a Complex with Tyrosine-phosphorylated Plakoglobin in the Membrane-associated Triton-soluble Pool of Proteins—After sequential detergent extraction, two higher molecular weight proteins (~160–180 kDa) co-immunoprecipitating with Pg were also detected by the phosphotyrosine antibody (Fig. 3, A, C, and E). At ~160 kDa, desmogleins, the transmembrane desmosomal cadherins, are well characterized binding partners for Pg (for review see Ref. 29). Therefore, we tested whether the most abundant desmoglein in A431 cells, desmoglein 2, represented one of these high molecular weight phosphorylated proteins found in the 11E4 immunoprecipitates. Triton-soluble lysates of A431 cells treated for 0, 5, or 20 min with EGF were processed for Pg immunoprecipitation. The immunoprecipitates were subjected to immunoblot analysis for phosphotyrosine confirming the presence of the two high molecular weight phosphorylated pro-
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Plakoglobin tyrosine point mutant constructs were used in order to identify the C-terminal tyrosine residues of Pg targeted by EGF-dependent phosphorylation. COS-7 cells were transfected with EGFR, Ecad-Dsg1, and either Pg F660 in which tyrosine residue Tyr-660 was substituted for a phenylalanine, Pg F693 in which tyrosine residue Tyr-693 was substituted for a phenylalanine, or Pg C deletion receptor (Tyr-660, Tyr-693, Tyr-701, Tyr-705, Tyr-724, Tyr-729, and Tyr-737). The Tyr-693 of the Pg Phe-693 point mutant has been substituted for a phenylalanine. The C-terminal tyrosine residues, Tyr-693, Tyr-724, and Tyr-729, were substituted for phenylalanines to generate the Pg Phe-693, Phe-724, Phe-729 point mutant construct. The triple lines represent the armadillo repeats found in Pg and its family members (48). All deletion and point mutant Pg constructs were C-terminally Myc epitope-tagged.

EGF is reported to interact with β-catenin (13) and we repeatedly detected a phosphorylated protein of a similar molecular weight to EGFR in Pg immunoprecipitation experiments (Fig. 3, A, C, and E), we hypothesized that the receptor associates with Pg to trigger its phosphorylation. To address this question, we carried out immunoprecipitations using antibodies directed against β-catenin or Pg from cells treated with EGF for 0 or 30 min. In addition, because Dsg2 was shown to co-immunoprecipitate with Pg (Fig. 4A), we tested whether immunoprecipitating Dsg2 would co-immunoprecipitate EGFR in a manner similar to the reported co-immunoprecipitations of EGFR with E-cadherin (30). Analysis of the resulting immunoprecipitates by blotting with an EGFR antibody showed that under the conditions used for these experiments, EGFR was not associated with any of the immunoprecipitated proteins. However, it was easily detected by direct EGFR immunoprecipitation (Fig. 4B). The membrane was stripped and reprobed for tyrosine-phosphorylated proteins revealing a high molecular weight band of similar molecular weight to EGF in all EGF-activated immunoprecipitates, including beads only and IgG controls. In the lane corresponding to the specific EGFR immunoprecipitation after EGF treatment, the signal was much stronger due to the presence of activated EGFR as shown in the EGFR blot (Fig. 4B). Therefore, we conclude that the higher of the two phosphorylated high molecular weight bands detected under these conditions in the Pg immunoprecipitations (Fig. 4A) was due to a nonspecific interaction of the Sepharose beads or IgG with a tyrosine-phosphorylated protein of a similar molecular weight to EGFR. Note that the phosphorylated specific band corresponding to Dsg2 is detected in both Pg and Dsg2 immunoprecipitates (Fig. 4B). In addition, co-immunoprecipitation of E-cadherin with β-catenin and β-catenin with E-cadherin was confirmed following stripping of the blot (data not shown).

To extend this analysis, A431 cells were serum-starved overnight and treated with EGF for 0, 5, or 20 min to reproduce conditions under which the β-catenin-EGFR interaction was detected previously (13). Cells were harvested in 0.5% Triton buffer, glycerol containing HEPES buffer to facilitate the maintenance of protein complexes during the immunoprecipitation procedures. The cell lysates were subjected to EGFR immunoprecipitation using Sepharose beads as a control. The immunoprecipitates were probed with an EGFR antibody first, and the membranes were subsequently stripped and reprobed for β-catenin, Pg, or the well characterized EGFR-binding adaptor protein Shc (Fig. 4C). Under these experimental conditions, no β-catenin nor Pg were detected in association with EGFR, although high amounts of receptor were immunoprecipitated as shown by the EGFR blot and Shc co-immunoprecipitated with EGFR (Fig. 4C).

Specific Tyrosine Residues of the C-terminal Domain of Pg Are Targeted by EGFR Activity—COS-7 cells transiently expressing N- or C-terminal truncations of Pg were analyzed to confirm the requirement of the C terminus of Pg for EGF-induced tyrosine phosphorylation. COS-7 cells were transfected with EGFR, Ecad-Dsg1, and PgAC or PgAN. The 9E10 anti-Mye antibody was used to immunoprecipitate the C-terminally tagged Pg deletion mutants (Fig. 5, Aa lanes 1, 2, 6, and 7). Slight phosphorylation of PgAC was detected in this system as compared with the high level of phosphorylation observed for PgAN (compare lanes 2 and 7, Fig. 5A), confirming that the C terminus of Pg is required for efficient EGF-induced tyrosine phosphorylation as indicated by the results obtained in A431LPGc cells (Fig. 3).

Plakoglobin tyrosine point mutant constructs were used in order to identify the C-terminal tyrosine residues of Pg targeted by EGF-dependent phosphorylation. COS-7 cells were transfected with EGFR, Ecad-Dsg1, and either Pg F660 in which tyrosine residue Tyr-660 was substituted for a phenylalanine or with Pg Phe-693, Phe-724, Phe-729 in which the three C-terminal tyrosine residues Tyr-693, Tyr-724, and Tyr-729 were mutated into phenylalanines (Fig. 2). After overnight serum starvation, cells were treated with EGF for 0 or 5 min and processed for immunoprecipitations with the 9E10 antibody. Although Myc-tagged Pg point mutants were both efficiently immunoprecipitated (Fig. 5B, Blot: Pg), Pg Phe-660 was phosphorylated in response to the 5-min EGF treatment, whereas Pg Phe-693, Phe-724, and Phe-729 showed no sign of EGF-dependent phosphorylation (compare lanes 2 and 5, Fig. 5B, Blot: PY). The lack of phosphorylation of Pg Phe-693, Phe-724, and Phe-729 was also confirmed after 24-h activation of the EGFR (data not shown). This indicated that Pg C-terminal residues Tyr-693, Tyr-724, and/or Tyr-729 are specific targets for EGF-dependent phosphorylation of Pg.

Pg Phosphorylated upon EGF Activation Is Associated with Dsg2 but Not with DP N-terminal Domain—A reported consequence for EGF-dependent phosphorylation of β-catenin is to modulate negatively the interaction of E-cadherin with the actin cytoskeleton (10). To explore whether the EGF-dependent phosphorylation of Pg can similarly affect the interaction of desmosomal components with the intermediate filament cytoskeleton, we tested the effect of Pg tyrosine phosphorylation on the interaction between Pg and its direct binding partners Dsg and DP (20). DP links the desmosomal plaque to intermediate filaments (19, 31–33). COS-7 cells were transiently transfected with EGFR, Dsg2, and Pg, and in parallel with EGFR, Ecad-Dsg1, Pg, and a FLAG epitope-tagged desmoplakin N-terminal polypeptide (DP-NTP) (19). DP-NTP binds Pg and is recruited to cell-cell borders in COS-7 cells in the presence of
Fig. 3. Subcellular distribution and tyrosine phosphorylation status of plakoglobin and plakoglobin deletion mutants. After 24 h of serum starvation, A431, A431.PgΔC, and A431.PgΔN cells were treated with 10 nM EGF for 0, 2, or 5 min followed by sequential detergent extraction and immunoprecipitation with the Pg antibody 11E4 or the polyclonal Pg C-terminal antibody for the PgΔN samples. Immunoprecipitates were run in duplicate, on SDS-PAGE gels, transferred to nitrocellulose, and immunoblotted with the 4G10 anti-phosphotyrosine antibody (A, C, and E) or the Pg N-terminal antibody 11E4 (B) or the Myc monoclonal antibody 9E10 (D and F). Although Pg could be detected in all three pools (B), tyrosine-phosphorylated Pg was predominantly in the Triton-soluble pool (A). Upper bands (~180 kDa) are discussed in the text and addressed in Fig. 4. PgΔC could be detected in all three pools using the Myc antibody (arrowhead in D) and was not tyrosine-phosphorylated in response to EGF treatment (arrowhead in C). Endogenous Pg from A431.PgΔC cells was still phosphorylated in response to EGF (asterisk in C). As described previously (22), the majority of PgΔN was detected in the cytosolic and Triton-soluble pools (F). Tyrosine-phosphorylated PgΔN (arrowhead in E) and endogenous Pg (asterisk in E) could be detected in both these pools. After 2 min of EGF treatment a transient decrease in the amount of PgΔN in the Triton-insoluble pool could be detected (F). The fold decrease in the Triton-insoluble pool of PgΔN, established by scanning densitometry, was 3.7 ± 1.8 (from three different experiments).

Pg and a desmosomal cadherin tail (20). Cells were serum-starved overnight and treated with EGF for 0 of 5 min or 24 h prior to 6D8 antibody or M2-agarose immunoprecipitations. The 6D8 antibodies precipitated Dsg2, whereas the M2 antibodies precipitated FLAG-tagged DP-NTP, as shown by the immunoblot probed with 6D8 and NW161, which is directed against the DP N terminus (Fig. 6, A and B, Blot Dsg2 and Blot DPNT). Pg was co-immunoprecipitated with Dsg2 independently of EGF treatment as shown by the 1407 blot (Fig. 6A, right panel, Blot Pg) and was efficiently phosphorylated as shown by the Pg immunoprecipitations (Fig. 6A, left panel, Pg IP). Likewise, Pg was co-immunoprecipitated with DP-NTP independently of EGF treatment as assessed with the 1407 anti-Pg antibody (Fig. 6B, Blot Pg) and was efficiently phosphorylated as shown by the Pg immunoprecipitations (Fig. 6B, Pg IP). However, no tyrosine-phosphorylated Pg was detected in association with DP-NTP after either short or long term EGF treatment (Fig. 6B, Blot PY), whereas phosphorylated Pg was detected in the Dsg2 immunoprecipitations (Fig. 6A, middle panel, blot PY). Interestingly, a high level of phosphorylation of Dsg2 was detected in these samples as shown by the upper band in the PY blot (Fig. 6A, middle panel). These findings suggest that EGFR activation promotes tyrosine phosphorylation of Pg, which remains associated with its transmembrane binding partner Dsg2, while it does not associate, in the cytosolic or membrane associated pools of proteins, with the N-terminal domain of its desmosomal plaque binding partner DP.

EGF-dependent Phosphorylation of Plakoglobin Prevents the Formation of DP-NTP and Plakoglobin Complexes—The results described in Fig. 6 indicate that in transiently transfected COS-7 cells, the Pg associated with DP-NTP after EGF treatment is not tyrosine-phosphorylated. These results, however, do not rule out that the Pg associated with DP-NTP is not available for phosphorylation by EGFR. To take a more direct approach to determine whether phosphorylation of Pg prevents its association in a complex with DP-NTP, in vitro techniques using recombinant Pg and DP-NTP were initiated. However, efforts were hampered due to technical problems including the inefficiency of in vitro phosphorylation of Pg by purified EGFR kinase. Consequently, we took a different approach, generating an A431 stable cell line inducible for DP-NTP. Our goal was to create a system in which Pg becomes tyrosine-phosphorylated prior to the synthesis of its DP binding partner. This would rule out the possibility that phosphorylated Pg is not associated with DP-NTP simply because this interaction makes Pg inaccessible for tyrosine phosphorylation. A time course of induction demonstrated that DP-NTP was detectable in the cell lysates only after ~12 h of induction with doxycycline (Fig. 7A), whereas we showed previously that Pg is phosphorylated in A431 cells as early as 2 min after EGF treatment (Fig. 3). Therefore, cells were treated with doxycycline and EGF for 24 h and subjected to parallel Pg and M2-agarose immunoprecipitations. Phosphorylated Pg was detected independently of doxycycline treatment (Fig. 7B, Pg IP). DP-NTP was only detected in the M2-immunoprecipitates derived from doxycycline-induced cells as shown by the DPNT blot (Fig. 7B). As expected, Pg was co-immunoprecipitated with the induced DP-NTP (Fig. 7B, blot Pg). However, no phosphorylated Pg was detected in the M2 immunoprecipitates (Fig. 7B, blot PY). These results indicate that newly synthesized DP-NTP is able to form new complexes with non-phosphorylated Pg, whereas it is unable to associate with tyrosine-phosphorylated Pg.
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Plakoglobin and β-catenin are substrates for tyrosine kinases and associate with tyrosine phosphatases suggesting...
sine phosphorylation in regulating protein-protein interactions among desmosomal components is unknown.

In the present study, we demonstrate that, like β-catenin, Pg becomes more detergent-soluble in scattering cells in response to long term EGF treatment (Fig. 1) suggesting a decrease in its association with the cytoskeleton. As reported previously for β-catenin (13), Pg is rapidly phosphorylated on tyrosine residues upon EGFR activation, and this tyrosine phosphorylation is dependent on 1 or more of 3 residues in its C terminus. Furthermore, tyrosine-phosphorylated Pg was predominantly found in the membrane-associated Triton-soluble pool (Fig. 3), indicating a similar localization to the receptor tyrosine kinase EGFR. We did not detect any tyrosine-phosphorylated Pg in the Triton-insoluble pool of proteins in either short (Fig. 3) or long (not shown) term EGF treatment, suggesting the possibilities that phosphorylated Pg is unable to integrate into junctions and/or that junctional Pg is not a substrate for EGFR tyrosine kinase activity. However, we cannot rule out the possibility that Pg may rapidly exchange out of the Triton-insoluble pool upon phosphorylation, thereby preventing its detection.

Fig. 6. Phosphorylated Pg is found in a complex with Dsg2 but not DP-NTP after EGFR activation. A, COS-7 cells were transiently transfected with EGFR, Dsg2, and Pg. After 24 h incubation at 37 °C, the cells were serum-starved overnight. Prior to immunoprecipitation (IP), cells were treated with 10 nM EGF for 0 or 24 h. 11E4 Pg immunoprecipitates were run on SDS-PAGE gels and transferred to nitrocellulose. The nitrocellulose blots were probed with the HRP-conjugated PY99 anti-phosphotyrosine antibody (PY), stripped, and reprobed with the 1407 Pg antibody (Pg). Significant phosphorylation of total Pg was observed following EGF treatment (left panels). Anti-Dsg2 6D8 immunoprecipitates, generated from the same cell lysates used for Pg immunoprecipitations, were run on SDS-PAGE gels, transferred to nitrocellulose, and immunoblotted with the HRP-conjugated PY99 anti-phosphotyrosine antibody (PY). The PY99 antibody was stripped off the nitrocellulose membrane, and the blots were reprobed with the 1407 Pg antibody (Pg). Phosphorylated Pg was easily detected co-immunoprecipitating with its well characterized binding partner Dsg2. The band denoted ns is nonspecific (middle panels). The strength of the phosphorylation signal obtained for Dsg2 (see middle panel) hindered the stripping of the PY99 antibody to efficiently reprobe the PY blot with Dsg2 Ab. Therefore, parallel 6D8 immunoprecipitates were run on SDS-PAGE gels and transferred to nitrocellulose. The top of the membrane was immunoblotted with anti-Dsg2 6D8 antibody, and the bottom was probed with 1407 Pg antibody. The ability of Dsg2 to associate with Pg was not altered by the tyrosine phosphorylation of both Pg and Dsg2 resulting from the 24-h EGF treatment (right panel). B, COS-7 cells were transiently transfected with EGFR, ECad-Dsg1, Pg, and FLAG epitope-tagged DP-NTP. After 24 h incubation at 37 °C, the cells were serum-starved overnight. Prior to immunoprecipitation, cells were treated with 10 nM EGF for 0 or 5 min or 24 h. Pg immunoprecipitations using the 9E10 Myc antibody were run on SDS-PAGE gels and transferred to nitrocellulose. The nitrocellulose blots were probed with the HRP-conjugated PY99 anti-phosphotyrosine antibody (PY), stripped, and reprobed with the 1407 Pg antibody (Pg). Significant phosphorylation of total Pg was observed following 5 min or 24 h of EGF treatment (left panels). Anti-FLAG M2-conjugated agarose beads immunoprecipitates generated from the same cell lysates used for Pg immunoprecipitations were run in duplicate, on SDS-PAGE gels, transferred to nitrocellulose, and immunoblotted with the HRP-conjugated PY99 anti-phosphotyrosine antibody (PY) and the NW161 DP N-terminal antibody (DPNT). The PY99 antibody was stripped off the nitrocellulose membranes, and the blots were reprobed with the 1407 Pg antibody (Pg). Although phosphorylated Pg was readily detected in the control experiment shown on the left, no phosphorylated Pg was ever found associated with the DP N-terminal polypeptide in the EGF-treated samples both after 5 min and 24 h of activation of the receptor tyrosine kinase (right panels). The blots presented in this figure are representative of five independent experiments.
subjected to a time course of induction. Cells were treated with 2 epitope-tagged DP-NTP expression, in response to doxycycline, were complexes.

A, were treated with 2 the poly-FLAG or 1407 blots. No phosphorylated Pg was found associ-

antibody. The exposure shown for the PY99 blot is twice as long as for 

off the membrane which was subsequently reprobed with 1407 Pg 

with transferred M2 immunoprecipitates were probed with poly-FLAG 

membranes with 11E4 immunoprecipitates were probed 

rose beads directed against the FLAG tag and the Pg antibody 11E4. 

were then processed for immunoprecipitation with M2-conjugated aga-

rose beads directed against the FLAG tag and the Pg antibody 11E4. 

Immunoprecipitates were run on SDS-PAGE gels and transferred to 

nitrocellulose. Membranes with 11E4 immunoprecipitates were probed with the PY99 antibody. After 24 h of EGF treatment, phosphorylated Pg was present regardless of doxycycline induction. The membranes with transferred M2 immunoprecipitates were probed with poly-FLAG and PY99 phosphotyrosine antibody. The PY99 antibody was stripped off the membrane which was subsequently reprobed with 1407 Pg antibody. The exposure shown for the PY99 blot is twice as long as for the poly-FLAG or 1407 blots. No phosphorylated Pg was found associated to DP-NTP.

in the junctional pool. Tyrosine-phosphorylated β-catenin was restricted to the Triton-soluble pool of proteins in ras-transformed cells (8) and was found associated to E-cadherin after growth factor treatment (13). Consistent with these observations we found that the Pg contained in the Triton-soluble pool was associated with Dsg2 after EGF treatment (Fig. 4A). Moreover, tyrosine-phosphorylated Pg remains associated with the desmosomal cadherin Dsg2 (Fig. 6). During this study we also detected tyrosine-phosphorylated desmoglein 2 in response to EGFR activation, and this may represent another regulatory step in the EGFP-dependent regulation of desmosomes.

Intriguingly, in a short time frame of growth factor-induced tyrosine phosphorylation, a transient but reproducible decrease in the Triton-insoluble pool of the deletion mutant PgΔN was observed after short term EGF treatment (Fig. 3F). Our ability to detect a decrease in the level of PgΔN, but not full-length Pg or PgΔC, in the Triton-insoluble pool may be due to the fact that levels of PgΔN found in this pool are already relatively low. Thus, it seems possible that the higher levels of Pg found in the Triton-insoluble pool could mask subtle changes in the distribution of a relatively minor population of tyrosine-phosphorylated full-length Pg in control cells after short term growth factor treatment. The rapid changes in the solubility observed for PgΔN in response to EGF may represent early events that normally occur during remodeling of intercellular junctions and acquisition of a motile phenotype. Taken together, the rapid phosphorylation of Pg and the reproducible detection of changes in its subcellular distribution after long term EGF treatment may indicate that the regulatory effects of EGFR kinase on full-length Pg are a multistep process, which evolves from the initial phosphorylation of Pg to eventually promote its subcellular redistribution, hence remodeling of desmosomes. An increase in the cytosolic pool of PgΔC was also observed, even though this truncated Pg molecule does not become tyrosine-phosphorylated. This raises the possibility that interactions with other partners that normally stabilize PgΔC in the membrane-associated pool may be compromised in an EGF-dependent manner (e.g. because they are EGFR substrates) by deletion of the Pg C terminus.

In A431.PgΔN and A431.PgΔC cells, endogenous tyrosine-phosphorylated Pg was not only detected in the membrane-bound Triton-soluble pool but also somewhat unexpectedly in the cytosolic pool (Fig. 3E). Furthermore, the majority of the phosphorylated deletion mutant PgΔN was found in the cytosolic pool (Fig. 3E). Possibly due to its increased metabolic stability through the deletion of its GSK3β phosphorylation site, which targets full-length Pg for degradation (38, 39), PgΔN accumulates in the cytosolic pool (22). Therefore, since catenins are thought to exchange between non-cadherin-associated and cadherin-associated pools (40), it is possible that tyrosine-phosphorylated PgΔN exchanges from the membrane-bound, Triton-soluble pool into the cytosolic pool where it is easily detected upon accumulation. This exchange might also be enhanced due to changes in stability in the membrane pool, as described above for PgAC. The presence of tyrosine-phosphorylated full-length Pg in the cytosolic pool may be due to its increased ability to associate with PgΔN and PgΔC (as reported previously (22)).

The transmembrane receptor tyrosine kinase EGFR was reported previously to associate both with its substrate β-catenin (13) as well as E-cadherin (30). In our experimental conditions, we did not detect a stable interaction between EGFR and components of either the classic or desmosomal cadherin-cate-

nin complex (Fig. 4). Although the reasons underlying the apparent discrepancy between the current results and those reported previously are unknown, it has been shown that the interaction between E-cadherin and EGFR is regulated by calcium-mediated cell contact and other factors that influence the presence of the receptor in the cadherin-catenin complex (30). Along with potential cell type-specific differences in the formation of these signaling complexes, differences in culture conditions and cell density could account for the variation in reported findings. The fact that plakoglobin is nevertheless robustly phosphorylated in an EGF-dependent manner in the present study is consistent with the possibilities that (a) labile or transient interactions between activated EGFR and the membrane-associated desmoglein-plakoglobin complex are sufficient to drive the observed tyrosine phosphorylation of these desmosomal proteins and/or (b) that a downstream non-recep-
tor tyrosine kinase is responsible for the observed tyrosine phosphorylation.

Several mechanisms have been proposed to explain the adhe-
sive changes associated with cadherin-catenin tyrosine phos-
phorylation (41). One model suggests that tyrosine phosphory-
atation of the cadherin-catenin complex leads to a decreased association of the complex with the cytoskeleton. Linkage of the classical cadherins to the cytoskeleton has been shown to be required for classical cadherin-mediated adhesion in epithelial cells (42, 43), and a decreased association with the cytoskeleton is thought to lead to decreased adhesion. It has also been
suggested that phosphorylation of junctional components other than β-catenin may be involved in the modulation of classical cadherin-based adhesion (44, 45). As Pg preferentially incorporates into desmosomes in cells that assemble both desmosomes and adherens junctions (17, 46, 47), Pg may play a particularly important role in cytoskeletal attachment and adhesive function in these junctions.

In addition to its ability to interact with desmosomal cadherins (21, 48–52), Pg has been shown to interact with the desmosomal plaque protein desmoplakin (20, 53, 54). Experiments conducted in transiently transfected COS-7 cells showed that upon short term EGFR activation, no interaction between tyrosine-phosphorylated Pg and the N-terminal domain of desmoplakin was detected (Fig. 6B). This finding raised the possibilities that the association of Pg in a complex with DP prevents its phosphorylation and/or that the phosphorylation of Pg inhibits its association with DP. These hypotheses were tested first by activating the EGFR kinase for 24 h, a time frame during which exchange between the different pools of proteins is expected to occur. The experiments still showed no DP associated with phosphorylated Pg in the membrane-associated Triton-soluble pool of proteins, consistent with the idea that phosphorylation of the catenin inhibits its interaction with the N-terminal domain of desmoplakin (Fig. 6B). Also supporting this interpretation are results from experiments carried out using A431 cells inducible for DP-NTP expression (Fig. 7). In these experiments pre-phosphorylated Pg was unable to participate in de novo interactions with the DP N terminus.

Together, these findings suggest that desmosomal protein interactions are affected by EGFR-dependent tyrosine phosphorylation in a manner comparable to that reported previously (10) for adherens junctions, that is the EGFR-dependent phosphorylation of catenins affects the linkage of the actin or intermediate filament cytoskeleton with the adherens junction or the desmosome components, respectively. As in adherens junctions, where the EGFR-dependent phosphorylation of β-catenin, Pg, and p120 does not affect their interaction with E-cadherin (10), the phosphorylation of Pg does not affect its interaction with the desmosomal cadherin Des2. Moreover, phosphorylated Pg does not associate with the desmosomal cytoskeletal linking protein DP. In this way, tyrosine phosphorylation of Pg could represent an early regulatory element of the EGFR-dependent cascade of events leading to the shift from an adhesive to a migratory phenotype necessary for epithelial cell motility.

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