Regulation of hemidesmosome disassembly by growth factor receptors

Coert Margadant, Evelyne Frijns, Kevin Wilhelmsen’, and Arnoud Sonnenberg

Division of Cell Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands. ’Present address: Department of Anesthesia and Perioperative Care, University of California, San Francisco, CA 94143.

ABSTRACT

Hemidesmosomes (HDs) promote stable adhesion of basal epithelial cells to the underlying basement membrane (BM). Critical for the mechanical stability of the HD is the interaction between the integrin α6β4 and plectin, which is destabilized when HD disassembly is required, for instance, to allow keratinocyte migration during wound healing. Growth factors such as epidermal growth factor (EGF) can trigger HD disassembly and induce phosphorylation of the β4 intracellular domain. Whereas tyrosine phosphorylation appears to mediate cooperation with growth factor signaling pathways and invasion in carcinoma cells, serine phosphorylation seems the predominant mechanism to regulate HD destabilization. Here, we discuss recent advances that shed light on the involved residues, the identity of the kinases that phosphorylate them, and the interactions that are targeted by these phosphorylations.

INTRODUCTION

Hemidesmosomes (HDs) are specialized multiprotein complexes that provide for stable adhesion of basal epithelial cells to the underlying basement membrane (BM) in (pseudo-) stratified as well as certain complex and simple epithelia (1). Two types of HDs can be distinguished on the basis of their components (Figure 1). Type II HDs are found in simple epithelia including that of the intestine, and consist of the integrin α6β4 and plectin (HD1). Type I (classical) HDs are found in (pseudo-) stratified epithelium, such as in the skin, and consist of α6β4, plectin, the tetraspanin CD151 and the bullous pemphigoid (BP) antigens 180 (type XVII collagen) and 230 (BPAG1) (1,2). Integrin α6β4 and BP180 bind with high and low affinity, respectively, to laminin-332 (Ln-332; previously called laminin-5) in the BM, and intracellular stabilization occurs via association of plectin and BP230 with keratin intermediate filaments, thus creating a stable anchoring complex (1-4). The importance of HDs in maintaining epithelial integrity is illustrated by two lines of evidence. Firstly, ablation of the genes encoding α6, β4, or plectin in mice results in severe blistering of the skin, causing neonatal death because of an epithelial barrier defect; however, knockout mice lacking BP180 or BP230 display only a mild form of skin blistering (1,2). Secondly, human patients carrying mutations in any of the HD components suffer from a skin blistering disorder known as epidermolysis bullosa. The severity of the disease depends on the type and location of the mutations, and their consequences at the mRNA and protein levels (5,6).

Despite the role of HDs in mediating stable adhesion, they are highly dynamic structures that can quickly disassemble under conditions where (partial) detachment from the BM is required, for example during cell division, differentiation or migration (7,8). Upon disassembly, HD components are no longer concentrated at the basal surface but are instead diffusely distributed over the plasma membrane or in the cytoplasm, or become translocated to lamellipodia (9-11). Although
the precise mechanisms that lead to HD disassembly remain obscure, it is at least partially triggered by, and dependent on, phosphorylation events of HD components elicited by growth factor stimulation. Phosphorylation of the β4 intracellular domain has been documented in response to hepatocyte growth factor (HGF), macrophage-stimulating protein (MSP), and primarily epidermal growth factor (EGF). However, significant controversies exist in the literature concerning the residues that are phosphorylated, their role in the regulation of HD destabilization, and the intracellular responses that are triggered by these phosphorylations independent of HD disassembly.

In this review, we first focus on protein-protein interactions governing HD assembly, and then discuss recent insights into how growth factor-induced phosphorylation events impact these interactions to regulate HD disassembly and α6β4-dependent functions in normal keratinocytes and carcinoma cells.

Figure 1 Schematic drawing of Type I and Type II HDs. Type II HDs are present in simple epithelia such as that of the intestine and consist solely of the integrin α6β4 and the plakin plectin (HD1). Type I HDs are found in (pseudo-)stratified epithelia such as that of the skin and additionally contain the tetraspanin CD151, the Type XVII collagen BP180, and the plakin BP230. BP230 and plectin mediate intracellular stabilization of the HD by binding to intermediate filament keratins. K, keratin; Ln, laminin.
PROTEIN-PROTEIN INTERACTIONS INVOLVED IN HD DISASSEMBLY

The cytoplasmic tail of β4A is 1017 amino acids long and consists of a membrane-proximal Na⁺-Ca²⁺ (CalX) exchanger motif and 2 pairs of fibronectin type III (FNIII) repeats, which are separated by a connecting segment (CS) (Figure 2). The cytoskeletal linker protein plectin can associate with either β4 or actin filaments, and these binding events are mutually exclusive (12-14). The interaction of the actin binding domain (ABD) of plectin with the first pair of FNIII repeats and the N-terminal 27 amino acids of the CS of β4 (residues 1115 – 1355) is thought to be the initial step in HD assembly, which is strengthened by additional interactions of the plectin plakin domain with the CS and the C-tail (Figures 2 and 3). Subsequently, BP180 interacts extracellularly with Ln-332, and intracellularly with plectin and the third FNIII repeat of β4. Lastly, BP230 is recruited through associations with BP180 and a region on β4 comprising the C-terminal 21 amino acids of the CS and the second pair of FNIII repeats (15-19). In addition to the multiple associations exerted by the cytoplasmic domain of the β4 subunit, the extracellular domain of the α6 subunit interacts with BP180 and CD151 (20). The crucial determinant for HD assembly is the interaction between β4 and plectin, as indicated both by the existence of type II HDs which can apparently form in the absence of BP180 and BP230 recruitment, and the hypoplastic nature of HDs that are observed in patients with mutations in β4 (R1281W or R1225H) that prevent this interaction (21,22). Furthermore, in vitro evidence indicates that disruption of the plectin-β4 interaction is sufficient to disrupt HD formation (17). It is therefore likely that HD disassembly in response to growth factor stimulation is primarily achieved by destabilization of the plectin-β4 interaction.

Figure 2 Structural organization of integrin α6β4 and plectin. Indicated are the various domains, the regions that are involved in the plectin-β4 interaction, and the positions of important tyrosine and serine phosphorylation sites as reported in the literature.
GROWTH FACTOR-INDUCED TYROSINE PHOSPHORYLATION OF THE β4-SUBUNIT

Several tyrosines located in the β4 cytoplasmic tail have been implicated in processes typically regulated by growth factor receptors (Figure 2). However, it is an area of many conflicting results. For instance, association of α6β4 with ErbB2 was reported in transformed keratinocytes, carcinoma cells, and ErbB2-transformed fibroblasts, resulting in ErbB2 autophosphorylation, activation of phosphatidylinositol 3-kinase (PI3-K), tumorigenesis and enhanced invasiveness (23-27). Activation of PI3-K and increased invasion were induced by ligation of α6β4, and the subsequent phosphorylation of primarily tyrosines 1257 and 1494 (28,29). Nevertheless, while one study determined Y1494 as the crucial residue, another study reported that the region spanning residues 854 to 1183 was required (27,29).

Association of α6β4 with the EGF receptor or c-Met (the HGF receptor) has also been reported in carcinoma cells (30-36). Upon stimulation with HGF, tyrosine phosphorylations in β4 elicit activation of both the PI3-K and extracellular signal-regulated kinase (ERK) pathways, leading to enhanced HGF-dependent tumorigenesis and invasion (33-35). Phosphorylation of Y1257, Y1440, Y1494, and Y1526 is responsible for coupling β4 to the Ras-ERK pathway, either via binding of Shp2 to β4, which results in the stimulation of Src and the subsequent phosphorylation of Gab1 on residues that promote Grb2 binding, or via the binding of Shc, which when phosphorylated also recruits Grb2 to the membrane (36,37). Cooperation between α6β4 and c-Met was independent of the extracellular domain, giving rise to the idea that the β4 cytoplasmic domain functions as a signaling platform for growth factor signaling pathways (33-36). However, a c-Met-α6β4 association was not detected by other researchers in the same cells. In addition, β4-enhanced invasion was not specific to c-Met, and c-Met could mediate invasion independently of β4 (38). Furthermore, the role of the β4 intracellular domain as a signaling adaptor is questioned by a recent study showing that β4-Shp2 association was only slightly increased by HGF, and HGF-induced invasion, as well as ERK and PI3-K signaling, were not enhanced by dimerization of the β4 intracellular domain (39). It therefore remains unclear exactly how β4 and c-Met cooperate in carcinoma cells.

The role of EGF-induced tyrosine phosphorylation events is also controversial; whereas initial studies suggested that phosphorylation of Y1422 and Y1440 in the CS of β4 mediates HD assembly, a later study by the same group confusingly reported the opposite, namely that these phosphorylations antagonize HD formation (37,40,41). A subsequent report then again implicated these residues in HD assembly based on the observation that phenylalanine substitutions impaired HD formation in an in vitro organotypic culture model (42). However, it remains ambiguous whether it is the inability to phosphorylate these residues or the mutations themselves that caused this effect. Given the available data, the contribution of tyrosine phosphorylation to HD disassembly under physiological conditions...
Regulation of hemidesmosome disassembly (i.e. in normal untransformed keratinocytes) is disputable. This is underscored by the observation that a β4 mutant that was not tyrosine-phosphorylated in response to EGF was not impaired in mediating EGF-stimulated migration and thus HD disassembly in keratinocytes (43). In addition, in normal keratinocytes as well as the same transformed cell lines used in the aforementioned studies, tyrosine phosphorylation was absent or only marginally detected by several groups, both in unstimulated conditions and under conditions when HDs are disassembled such as during EGF stimulation. Instead, serine phosphorylation of β4 was evident under steady state conditions, and increased in the presence of EGF (44-47).

Altogether, the functional relevance of tyrosine phosphorylation of the β4 cytoplasmic domain may be restricted to processes such as carcinoma invasion. Association of α6β4 with a growth factor receptor and tyrosine phosphorylation of β4 in carcinoma cells may represent aberrant phenomena that are induced by overexpression of growth factor receptors or the constitutive signaling by hyperactive receptor tyrosine kinases, as commonly observed in transformed cells. Moreover, the HDs in carcinoma cells are often rudimentary and structurally inferior due to decreased expression levels of BP180 and

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**Figure 3 Hypothetical models for HD disassembly induced by serine phosphorylation.** When not phosphorylated, the β4 intracellular domain interacts with the ABD and the plakin domain of plectin. Upon serine phosphorylation of the β4 CS, binding of the plectin ABD is prevented either by (A) a conformational change leading to intramolecular folding of the β4 cytoplasmic domain, or (B) binding of an alternative protein to the phosphorylated CS of β4.
BP230, and α6β4 localization is no longer confined to the basal surface but is in fact diffusely distributed over the membrane, which may increase its susceptibility for active kinases (2,48).

**GROWTH FACTOR-INDUCED SERINE PHOSPHORYLATIONS OF HD COMPONENTS**

Early reports documented a redistribution of HD components from the basal surface to the cytosol upon phorbol ester-induced activation of members of the protein kinase C (PKC) family of serine/threonine kinases, suggesting that PKCs regulate HD disruption (49). This was confirmed in later studies demonstrating the breakdown of HDs in carcinoma cells and normal keratinocytes after activation of PKC-family members or overexpression of PKC isoforms. In particular PKCα and PKCδ have been implicated in this process, with the specific isoform involved seemingly cell type-dependent (44-47).

The β4 cytoplasmic domain is phosphorylated on serines under steady-state conditions, which is augmented after phorbol myristate acetate-stimulated PKC activation or, physiologically more relevant, EGF stimulation. Serine phosphorylations occur primarily in the CS and the C-tail, and phosphopeptide mapping experiments identified S1356, S1360, and S1364 in the CS as the most prominent sites (Figure 2) (46,47). They are embedded in an amino acid context that is highly conserved, in mammals as well as in evolutionarily more distant species such as fish (Table 1), which suggests a critical role for this region. Indeed, studies using mutants carrying either phosphomimic aspartic acid or non-phosphorylatable alanine substitutions pointed out that phosphorylation of two or more of these serines prevents binding of the plectin ABD to β4. Accordingly, β4 mutants with triple aspartic acid substitutions were significantly impaired in HD formation under steady-state conditions, whereas mutants carrying triple alanine substitutions formed robust HDs which were resistant to EGF-induced disruption (47). Although PKC is undoubtedly involved, it may not account for the phosphorylation of all three residues. Whereas in one study it was reported that at least two of the three moieties were PKC targets, we found that S1360 is the only PKC site on β4, at least in keratinocytes (46,47). In search for additional kinases involved, S1364 was identified as a site for protein kinase A (47). However, there is no evidence for protein kinase A activation downstream of the EGF receptor in keratinocytes, whereas the EGF-induced activation of PKC is well established. The exact identity of all kinases triggering β4 serine phosphorylations in response to EGF remains to be determined.

Interestingly, S1356, S1360 and S1364 are not directly involved in plectin binding, and are located in a region that can be deleted without compromising HD formation (15,18). There is evidence suggesting that the C-tail of β4 can bind intramolecularly to a 321 amino acid segment including the first pair of FNIII repeats and part of the CS (16,19). These regions of β4 also bind to a segment of the plectin...
plakin domain (17), thereby enforcing the interaction between the two proteins. Possibly, this complex is disrupted upon serine phosphorylation of β4, allowing two of the three phosphorylated serines to interact with arginines 1225 and 1281 in the second FNIII repeat (Figure 3). Since the arginines are essential for plectin binding (21,22), the segment of the CS containing the phosphorylated serines thus competes for binding with plectin. Alternatively, β4 phosphorylation may increase its affinity for a third protein, that when bound to β4 prevents plectin binding by steric hindrance (Figure 3) (1).

Although the main determinant for HD stability is the plectin-β4 interaction, additional associations must be broken for full HD dissolution, including the interactions of β4 with both BP180 and BP230. In this respect, it is noteworthy that BP180 is also phosphorylated by PKC, leading to its translocation out of HDs (49). It is conceivable that other HD components are subject to a similar mode of regulation. In fact, PKC-mediated phosphorylation of α6 has also been reported (45). Moreover, whereas the emphasis has been on the effects of EGF, it should be noted that EGF alone does not induce complete HD disruption. It is likely that in an in vivo situation such as during wound healing, additional growth factors known to modulate keratinocyte migration and proliferation induce the activity of other kinases that contribute to HD disassembly. These factors may

| Table 1 Sequence conservation of the region containing serines 1356, 1360 and 1364. |
|---------------------------------|-----|-----|-----|
| Homo sapiens (human)           | D V L R S P S G S Q R P S V S D D |
| Pan troglodytes (chimpanzee)   | D V L R S P S G S Q R P S V S D D |
| Pongo pygmaeus (orangutan)     | D V L R S P S G S Q R P S V S D D |
| Equus caballus (horse)         | D V L R S P A G S Q R P S V S D D |
| Bos taurus (cow)               | D V L R S P A G S Q R P S V S D D |
| Canis familiaris (dog)         | D V L R S P A S S Q R P S V S D D |
| Mus musculus (mouse)           | D V L R S P A S S Q R P S V S D D |
| Rattus norvegicus (rat)        | D V L R S P A S S Q R P S V S D D |
| Ochotona princeps (American pika) | D V L R S P A G S Q R P S V S D D |
| Monodelphis domestica (opossum) | D V L R S P A G S Q R P S V S D D |
| Erinaceus europaeus (hedgehog) | D V L R S P A G S Q R P S V S D D |
| Echinops telfairi (hedgehog tenrec) | D V L R S P A G S Q R P S V S D D |
| Gallus gallus (chicken)        | D V L R T P A G S K R P S V S D D |
| Danio rerio (zebrafish)        | E V L R S P T S S L R P S V T D L |
| Tetraodon nigroviridis (green spotted pufferfish) | E V L R S P T S S K S P S V S G D |
| Takifugu rubripes (Japanese pufferfish) | E V L K S P T G S K T P S V S G D |
| Oryzias latipes (Japanese ricefish) | D V L R S P S S S K A P S V S G D |
| Gasterosteus aculeatus (three-spined stickleback) | E V L R T P T G S K T P S V S G D |
include MSP and transforming growth factor-α and -β. For the latter factors, no evidence exists to date with respect to their involvement in HD disassembly, but an interesting report has highlighted the role of MSP, a ligand for the receptor tyrosine kinase Ron, in the breakdown of HDs. MSP-Ron signaling regulates multiple processes in keratinocytes including proliferation, survival, and migration. Keratinocyte stimulation with MSP results in the serine phosphorylation of α6β4, causing 14-3-3 protein-dependent mobilization to lamellipodia where it associates with Ron, and the partial breakdown of HDs (50). Furthermore, although the role of S1356, S1360 and S1364 is emphasized, they are not the only serines phosphorylated. Phosphorylation of additional serines on β4 may play a role to achieve full HD destabilization. The complete dissolution of HDs is likely to be the result of the concerted efforts of multiple kinases activated by distinct extracellular stimuli.

CONCLUSIONS

We have discussed recent findings on the mechanisms of HD disassembly by growth factor receptors, both in normal keratinocytes and carcinoma cells. The mechanisms involved may differ on the cell type investigated: tyrosine phosphorylation seems to mediate activation of growth factor signaling pathways involved in migration and invasion in carcinoma cells, while serine phosphorylation appears more relevant under physiological conditions in normal keratinocytes to destabilize HDs. It is possible that serine phosphorylation is also the primary mechanism to disrupt HDs in carcinoma cells, which then releases β4 to become phosphorylated on tyrosines. Serine phosphorylations primarily target the plectin-β4 interaction and may result in an intramolecular binding of the β4 cytoplasmic domain which prevents the interaction with plectin. Alternatively, a third protein may bind β4 when phosphorylated, thus preventing plectin binding through competition. Though an important role is established for EGF-induced PKC activation, it does not account for complete HD disassembly. Additional kinases and extracellular stimuli governing complete HD dissolution remain to be identified.

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ABBREVIATIONS

ABD, actin-binding domain; BM, basement membrane; BP, bullous pemphigoid; CS, connecting segment; EGF, epidermal growth factor; ERK, extracellular signal regulated kinase; FNIII, fibronectin-type III; HD, hemidesmosome; HGF, hepatocyte growth factor; Ln-332, laminin-332; MSP, macrophage-stimulating protein; PI3-K, phosphatidylinositol 3-kinase; PKC, protein kinase C
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