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Cytoplasmic salt bridge formation in integrin αvß3 stabilizes its inactive state affecting integrin-mediated cell biological effects

Martina A. Müller [a], Leonora Brunie [a], Anne-Sophie Bächer [a], Horst Kessler [b,c], Kay-Eberhard Gottschalk [d], Ute Reuning [a,*]

[a] Clinical Research Unit, Dept. for Obstetrics & Gynecology, Technische Universitaet München, Munich, Germany
[b] Institute for Advanced Study and Centre of Integrated Protein Science, Department Chemie, Technische Universitaet München, Garching, Germany
[c] Chemistry Department, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia
[d] Dept. of Experimental Physics, University of Ulm, Ulm, Germany

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ABSTRACT

Heterodimeric integrin receptors are mediators of cell adhesion, motility, invasion, proliferation, and survival. By this, they are crucially involved in (tumor) cell biological behavior. Integrins trigger signals bidirectionally across cell membranes: by *outside-in*, following binding of protein ligands of the extracellular matrix, and by *inside-out*, where proteins are recruited to ß-integrin cytoplasmic tails resulting in conformational changes leading to increased integrin binding affinity and integrin activation. Computational modeling and experimental/mutational approaches imply that associations of integrin transmembrane domains stabilize the low-affinity integrin state. Moreover, a cytoplasmic interchain salt bridge is discussed to contribute to a tight clasp of the α/ß-membrane-proximal regions; however, its existence and physiological relevance for integrin activation are still a controversial issue. In order to further elucidate the functional role of salt bridge formation, we designed mutants of the tumor biologically relevant integrin αvß3 by mutually exchanging the salt bridge forming amino acid residues on each chain (αv_{R995D} and $ß3_{D723R}$). Following transfection of human ovarian cancer cells with different combinations of wild type and mutated integrin chains, we showed that loss of salt bridge formation strengthened αvß3-mediated adhesion to vitronectin, provoked recruitment of cytoskeletal proteins, such as talin, and induced integrin signaling, ultimately resulting in enhanced cell migration, proliferation, and activation of integrin-related signaling molecules. These data support the notion of a functional relevance of integrin cytoplasmic salt bridge disruption during integrin activation.

1. Introduction

One major group of adhesion and signaling receptors is represented by the integrin superfamily. These heterodimeric adhesion receptors are composed of two non-covalently linked subunits, the α- and the β-chains. Each subunit consists of a large extracellular domain, a transmembrane domain (TMD) and a relatively short cytoplasmic tail, connecting the integrin with the actin cytoskeleton [1,2]. Integrins recognize proteins of the extracellular matrix (ECM) e.g. via the tripeptide motif RGD which binds to the ligand binding site constituted by the two extracellular integrin head groups. Instrumental for integrin tasks is a fine-tuned regulation of their ligand binding affinity and cell signaling capability which is propagated into two directions across cellular membranes, *outside-in* and *inside-out*. Hereby, distinct biological events are provoked: during *inside-out* signaling, proteins such as talin, are recruited to ß-integrin cytoplasmic tails resulting in altered integrin conformation, including the ligand binding ectodomain, which increases integrin binding affinity; by *outside-in* signaling, integrins transmit signals into cells following ECM ligand binding. This also leads to conformational changes, enhancement of integrin clustering into focal adhesion contacts, and adaptation of cell adhesive behavior. This enables transduction of forces by integrins, cytoskeletal rearrangements, and altered gene expression profiles as a prerequisite for cell migration, proliferation, and survival [3–14].

The exact contribution of integrin TMD and cytoplasmic structural features to integrin activation is in many aspects still a matter of debate due to inconsistent findings. Regarding the cytoplasmic region, a putative membrane-proximal interchain salt bridge constituted between opposing charges of two conserved amino acid residues of the α- (GFFKR) and the ß- subunit (KLLITIHD) had been postulated (Fig. 1A) [7,13,15]. By nuclear

Abbreviations: ECM, extracellular matrix; TMD, transmembrane domain; VN, vitronectin; wt, wild type.

* Corresponding author at: Dept. for Obstetrics & Gynecology, Clinical Research Unit, Technische Universitaet Muenchen, Ismaninger Str. 22, D-81675 Munich, Germany. Tel.: +49 89 4140 7407; fax: +49 89 4140 7410.
E-mail address: ute.reuning@lrz.tum.de (U. Reuning).

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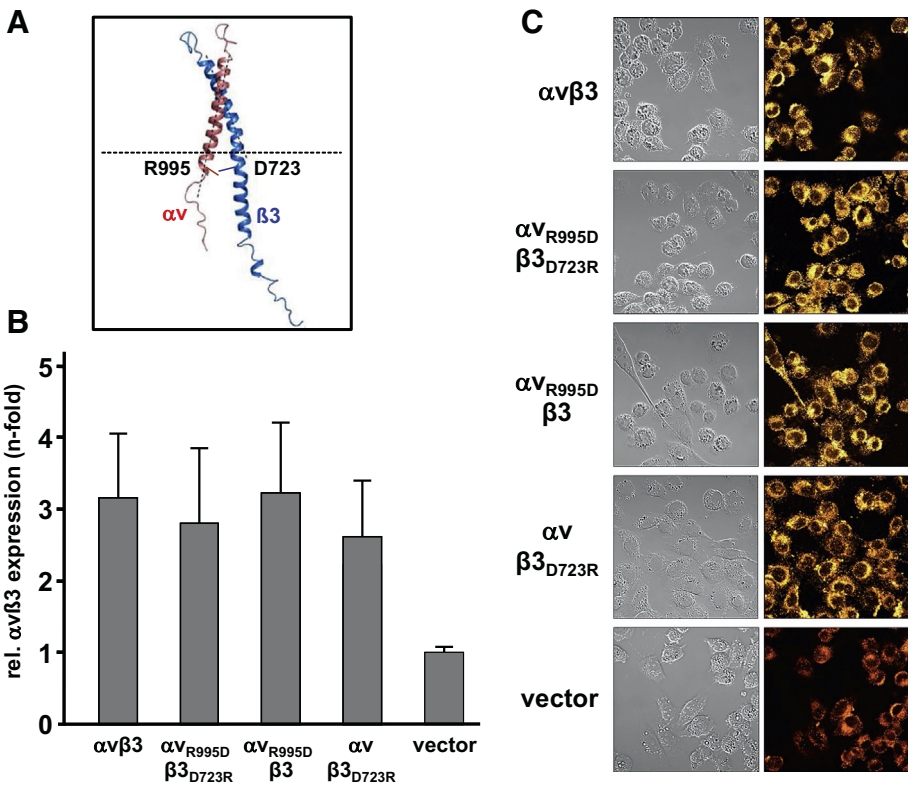

Fig. 1. Expression of αvß3 and its salt bridge mutants in stably transfected cell clones. (A) Scheme indicating the two salt bridge forming amino acid residues in the cytoplamic tail regions (αv_{R995} and $ß3_{D723}$). (B) Evaluation of αvß3 expression by FACS analysis. Cells transfected with αvß3-wt, $\alpha v_{R995D}ß3_{D723R}$ (charge reversal), $\alpha vß3_{D723R}$, $\alpha v_{R995D}ß3$, or empty expression vector were processed for αvß3 detection by FACS analysis as described. Values for FACS mean fluorescence intensities of three independent experiments (± s.d.) are given. (C) For immunocytochemical detection of αvß3, the above mentioned αvß3 transfectants were stained by the mAb # 23C6 which recognizes the ligand binding domain. Staining intensity was recorded by CLSM using the Zeiss Axiovert microscope. Depicted are representative fluorescence images and the corresponding differential interference contrast images.

magnetic resonance (NMR) spectroscopy using purified integrin cytoplasmic tails in aqueous solution, Vinogradova *et al.* [15] observed an electrostatic interaction between αIIb_{R995} $ß3_{D723/E726}$. These findings were, however, not confirmed in studies by others [16,17] where cytoplasmic integrin tails behaved like unstructured flexible domains lacking detectable interactions. These inconsistent NMR data argue—if at all—for a weak α/ß-cytoplasmic tail association at least in aqueous solution. Then later, NMR structural data on the αIIbß3 TMD including short membrane-proximal cytoplasmic stretches strongly suggested the existence of a salt bridge crucial for keeping integrins in a resting low-affinity state [7,13]. This stabilizing function of the salt bridge was verified by earlier *in vitro* studies of the integrins αIIbß3 and α5ß1, in which its mutational disruption promoted constitutive integrin signaling [18]. In the low-affinity conformation, at the transition to the cytoplasmic tails, integrin TMDs also have to adopt a clasped conformation which is inevitable for the formation of a cytoplasmic salt bridge.

The physiological relevance of salt bridge formation was examined *in vivo* using transgenic mice carrying a point mutation of the salt bridge-forming amino acid residue in the ß1-subunit ($ß1_{D759A}$). Most interestingly, these mice did not reveal any obvious phenotype. Moreover, primary keratinocytes originating from these mice displayed an unaltered adhesive, spreading, and migratory behavior when compared to wild-type (wt) cells. Consequently, it was argued that the putative salt bridge apparently lacks functions *in vivo* [19]. In clear contrast to this, in another mouse model, it was documented that salt bridge disruption in the integrin α4ß7 cytoplasmic tail (α4 R/AGFFKR) changed cell adhesion dynamics and destabilized the non-adhesive integrin conformation, thereby fostering leukocyte adhesion and migration [20].

Considering these contradictory results, it is reasonable to study the still elusive functional role of the interchain salt bridge in integrin activation and signaling. To this end, we designed mutants of the integrin αv- and ß3-subunit by exchanging the salt bridge forming amino acid residues (αv_{R995D} and $ß3_{D723R}$) and established a cellular test system by transfection of human ovarian cancer cells with different combinations of wt and mutant integrin chains: i) those capable of forming a salt bridge (αv-wt/ß3-wt or the charge reversal situation $\alpha v_{R995D}/ß3_{D723R}$) and ii) those lacking putative salt bridge formation (αv_{R995D}/ß3-wt or αv-wt/$ß3_{D723R}$). Herewith, we documented that loss of salt bridge formation enhanced αvß3-mediated cell adhesion to vitronectin (VN) and, moreover, constitutively activated integrin signaling and (tumor) cell biological events arising thereof.

2. Materials and methods

2.1. Materials

Dulbecco's modified eagle medium (DMEM), lipofectin™, fetal calf serum (FCS), geneticin G418, the plasmid pcDNA3.1/myc-His, and Alexa 488-conjugated goat-anti-mouse and goat-anti-rabbit IgG were purchased from Invitrogen, Carlsbad, CA, USA. The ECL™ chemiluminescent substrate and the BCA™ protein assay kit were obtained from Pierce, Rockford, IL, USA. Human VN, monoclonal antibodies (mAb) directed to FAK/FAK (pY397) were from Becton-Dickinson, Heidelberg, Germany. Monoclonal Ab raised against αvß3, αv, ß3, or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Millipore, Schwalbach, Germany. Monoclonal Ab directed to (p-)p44/$42^{(erk-1/2)}$, (p-)PKB/Akt, or polyclonal Ab to p-paxillin (Y118) were obtained from NEB Cell Signalling Technologies, Frankfurt, Germany. The polyclonal Ab directed to talin was purchased from Atlas Antibodies, Stockholm, Sweden. Poly-D-lysine (PL) and 4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide thiazolyl blue (MTT) were