IGF1 Binding to Integrin α6β4 Leads to Binding of the Calx-β Domain to Non-Catalytic (Allosteric) Site of IGF1R Kinase and Enhances Cell Survival

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Article

IGF1 Binding to Integrin α6β4 Leads to Binding of the Calx-β Domain to Non-Catalytic (Allosteric) Site of IGF1R Kinase and Enhances Cell Survival

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Abstract

Previous studies showed that IGF1 binds to α6β4 and induces α6β4-IGF1-IGF1R complex, which leads to the IGF1R kinase activation. An IGF1 mutant defective in integrin binding was defective in signaling and ternary complex formation, and acted as an antagonist, although the mutant still bound to IGF1R, suggesting that IGF1 binding to α6β4 plays a critical role in IGF1R activation. β4 has a unique long tail (>1000 residues) and deletion of part of the β4 tail containing the calx-β domain is known to reduce cell proliferation. We thus hypothesized that calx-β is involved in IGF1 signaling. Docking simulation predicted that calx-β binds to IGF1R kinase. We discovered that isolated calx-β bound to the IGF1R kinase domain. Point mutations in the predicted calx-β binding site in IGF1R kinase inhibited calx-β binding to IGF1R kinase. Notably, the isolated calx-β domain with cell-penetrating peptide (Tat-calx-β) enhanced survival of keratinocytes and non-transformed cells without IGF1. Tat-calx-β did not enhance survival of cancer cells. Several missense mutations are clustered in the predicted IGF1R kinase binding site of the calx-β domain of β4 in genetic skin blistering disease (JEB-PA). We discovered that several JEB-PA mutants were defective in calx-β binding to the IGF1R kinase and inhibited cell survival of keratinocytes, suggesting that these mutations may suppress calx-b binding to IGF1R kinase. These findings suggest that IGF1 binding to α6β4 triggers calx-β binding to the IGF1R kinase and activates IGF1R kinase.

Keywords: integrin α6β4; IGF1: calx-β domain; cell survival; IGF1R; JEB-PA

Introduction

The type I Insulin Like Growth Factor Receptor (IGF-1R) signaling pathway is a major oncogenic signaling pathway [1]. The elucidation of the IGF1 signaling pathway should have a major impact in designing new therapeutic strategies [2]. Many cancer cells secrete abnormally high levels of IGF1 and IGF2. Once released by cancer cells, both growth factors bind and activate IGF1R on the surface. IGF1R has been implicated in cancer progression [2]. Overexpression of IGF1R has been reported in multiple human cancer lineages and is associated with poor prognosis of cancer patients [3–5]. It has been proposed that IGF1 binding to IGF1R induces phosphorylation of specific tyrosine residues of IGF1R. These phosphotyrosines then bind to adapter molecules such as Shc and insulin receptor substrate (IRS)-1. Phosphorylation of these proteins leads to activation of the phosphoinositide 3-kinase (PI-3K) and mitogen activated protein (MAP) kinase signaling pathways [6]. This receptor activation causes the release of intracellular signals that are strongly anti-apoptotic, notably through their ability to activate the PI-3K/AKT pathway. Thereby IGF1 confers resistance to chemotherapy and radiation therapy.

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Integrins are a family of cell adhesion receptors that recognize extracellular matrix ligands (e.g., laminin and fibronectin) and cell surface ligands (e.g., ICAM-1 and VCAM-1) and soluble ligands (e.g., cytokines). Integrins are transmembrane α-β heterodimers, and at least 18 α and 8 β subunits are known [7,8]. In previous studies, we showed that IGF1 binds to integrins αvβ3 [9] and α6β4 [10] and induces integrin-IGF1-IGF1R ternary complex. Mutation of αvβ3-binding site in IGF1 (the R36E/R37E mutation) suppressed IGF1 signaling although the IGF1 mutant still binds to IGF1R. The role of integrins in IGF1 signaling was unclear. We recently discovered that IGF1 binding to αvβ3 induces the binding of Gα13 binding to the β3 tail through the EEE motif and blocking the binding of Gα13 to the β3 tail by mutating the EEE motif in the β3 tail suppressed IGF1 signaling, suggesting that Gα13 binding to the β3 tail is involved in IGF1 signaling [11]. Gα13 binding to the β3 tail is known to induce activation of RhoA [12], but it is unclear if any other downstream effectors of Gα13 exists. We predicted that Gα13 directly binds to the IGF1R kinase, and the location of the IGF1R kinase-binding site of Gα13 by docking simulation, and Gα13 mutant defective in IGF1R binding acts as an antagonist of IGF1 signaling. The predicted Gα13-binding site in IGF1R kinase is a non-catalytic site (designates the allosteric site). Gα13/IGF1R kinase interaction may be a novel therapeutic target in cancer.

Integrin α6β4 binds to laminins. α6β4 in normal epithelial cells is located in basal surface of the cells as a component of junctional complexes named hemidesmosomes that mediate stable adhesion to the underlying basement membrane [13,14]. α6β4 is up-regulated in various types of cancer [15]. α6β4 expression is correlated with the progression and metastatic potential of several different tumors [16–18]. α6β4 is relocated to the apical region of the cells in transformed cells [15]. α6β4 is shielded from growth factors in the basal surface of the cells, but when it moves to the apical region, α6β4 is exposed to growth factors. It has been proposed that α6β4 associates with growth factor receptors and this association plays an important role in the pathogenesis of diseases (e.g., cancer). We previously showed that α6β4 binds to IGF1 and induces α6β4-IGF1-IGF1R ternary complex formation on the cell surface [10]. Notably, α6β4 expression markedly enhanced IGF1-induced cell survival. IGF1 mutant defective in α6β4 binding (R36E/R37E) was defective in signaling and ternary complex formation, and acts as a dominant-negative antagonist, suggesting that α6β4 binding to IGF1 is critical for IGF signaling. The role of α6β4 in IGF1 signaling is not fully understood.

The integrin β4 subunit has a long (>1000 amino acid) cytoplasmic domain, which is distinct from that of other β subunits. Particularly, β4 has no EEE motif (Gα13-binding motif) and Gα13 may not be involved in IGF1 signaling. The cytoplasmic tail of β4 acts as a protein–protein interaction platform both in cell adhesion and signaling events [19]. Deletion of β4 tail (tail-less) induces proliferative defect in mice [20], suggesting that interactions mediated by the β4 tail are crucial for proper cell-cycle control and proliferative signaling. The Src-family kinase Fyn associates with α6β4 in response to EGF stimulation, leading to phosphorylation of the β4 subunit. The association with Fyn requires the transmembrane-proximal region of β4, residues 854–1183, which contains the calx-β domain (residues 989–1107) [21]. However, it is unclear if the calx-β domain participate in IGF1 signaling.

We discovered that the calx-β domain of β4 directly binds to IGF1R kinase by docking simulation and binding assays. Point mutations in the predicted calx-β binding site in the IGF1R kinase domain inhibited calx-β binding to IGF1R kinase. Also, several point mutations in a genetic disease junctional epidermolysis bullosa with pyloric atresia (JEB-PA) are clustered in the predicted IGF1R-binding site in the calx-β domain and suppressed calx-β binding to IGF1R. These findings are consistent with the docking model. Notably, calx-b binding site in IGF1R kinase is a non-catalytic site (allosteric site) and overlaps with the Gα13-binding site. We propose that IGF1 binding to α6β4 triggers the binding of calx-β to IGF1R kinase and induce cell survival and proliferation. Furthermore, the isolated calx-β domain with cell-penetrating peptide (Tat-calx-β) markedly enhanced survival of several cell types including keratinocytes without IGF1 in serum-free and anchorage-independence, suggesting that calx-β binding to IGF1R kinase is sufficient to induce cell

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survival and proliferation. Taken together, we propose that calx-β of β4 plays a critical role in IGF1 signaling.

Results

Docking Simulation Predicts That the Calx-β Domain Binds to IGF1R Kinase

Deletion of the β4 tail containing the calx-β domain abrogates the association of β4 with Fyn [21], it is likely that the calx-β domain may bind to Fyn and other kinases. We hypothesized that the calx-β domain may binds to the IGF1R kinase, since IGF1R kinase is predicted to be physically close to the β4 cytoplasmic domain in the *α*6β4-IGF1-IGF1R ternary complex [10]. We performed docking simulation of interaction between the calx-β domain and IGF1R kinase using Autodock3. Final docking poses were clustered (RMD<2 A) and the first cluster contains a docked pose (docking energy -18.4 kcal/mol) that is a likely pose that binds to IGF1R kinase (Figure 1a). The predicted calx-β/IGF1R kinase complex is shown in Figure 1b. Amino acid residues in the predicted binding interface is shown in Table 1. To study if the isolated calx-β domain binds to the IGF1R kinase domain in ELISA-type binding assays, wells of 96-well microtiter plate were coated with the isolated calx-β and incubated with IGF1R kinase (fused to GST) and bound kinase domain was detected using anti-GST. The IGF1R kinase domain bound to Immobilized calx-β domain in a dose-dependent manner, but control GST did not, suggesting that the IGF1R kinase binds to the calx-β (Figure 1c) as predicted.

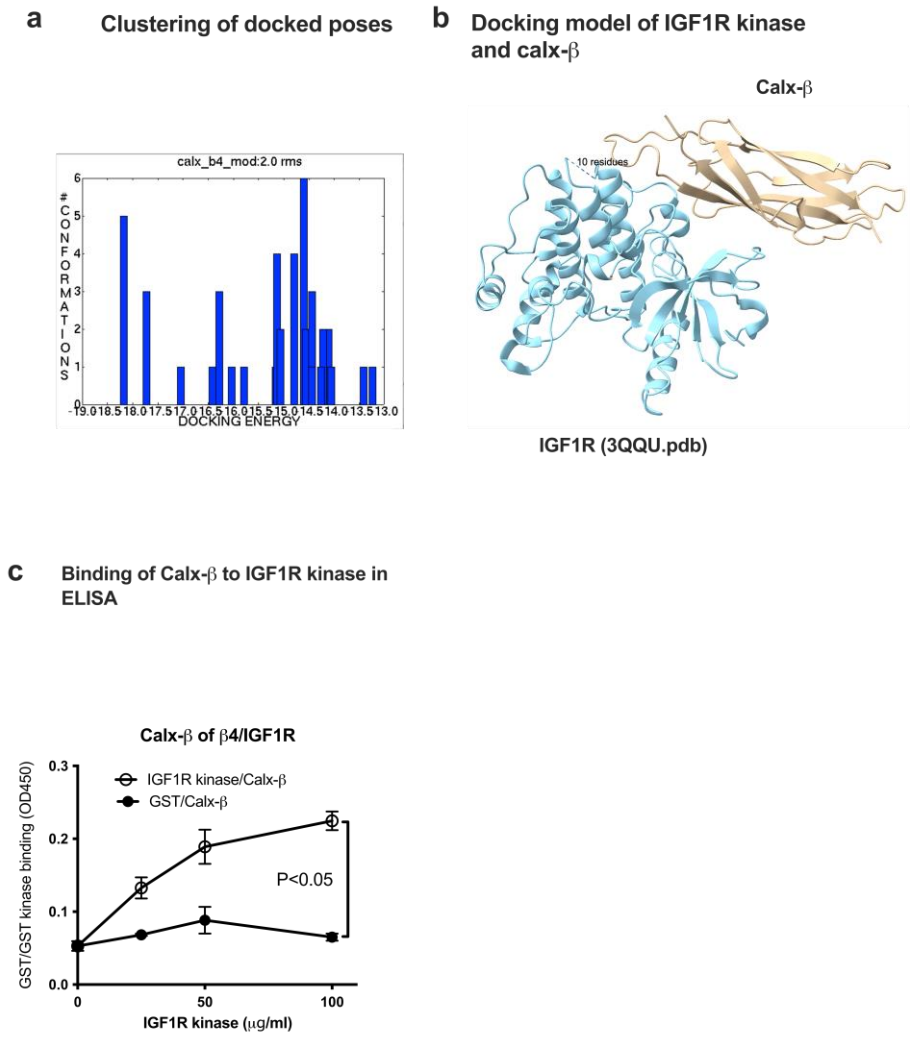

Figure 1. Docking simulation predicts that the calx-β domain binds to IGF1R kinase, and this prediction was confirmed by ELISA. (a) Clustering of docked models. Calx-β (from Swiss model depository) and IGF1R kinase

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(PDB code 3QQU) were used. Docked poses were clustered (RMS< 2A). The first cluster (Docking energy= -18.4 kcal/mol) represent the model in which calx-β most stably binds to IGF1R kinase. (b) Docking model of calx-β binding to IGF1R kinase. The simulation predicts that 1) IGF1R kinase binds to calx-β well, and 2) the binding interface is not in the catalytic sites of either molecule. (c) calx-β binding to the IGF1R kinase in ELISA. Wells of 96 well microtiter plate were coated with recombinant calx-β (1 μg/mL) and incubated with the IGF1R kinase (fused to GST). Control GST did not bind to calx-β. The data suggest that the IGF1R kinase bind to calx-β in a dose dependent manner. Data is shown as means +/- SD of triplicate experiments.

Point Mutations in the Predicted Calx-β Binding Site in IGF1R Kinase Suppress the Interaction

We introduced point mutations in the predicted calx-β-binding interface (residues 1020-1026) in the IGF1R kinase (Figure 2a). The mutations effectively suppressed calx-β binding to IGF1R kinase (Figure 2b). The minimum 3A mutation (K1020A/D1021A/E1022A) effectively inhibited calx-β binding to IGF1R kinase and further mutations (4A and 7A) did not enhance inhibition (Figure 2b). These findings are consistent with the docking model. Notably, the calx-b-binding site overlaps with the recently identified Gα13-binding site in IGF1R kinase (allosteric site) [11].

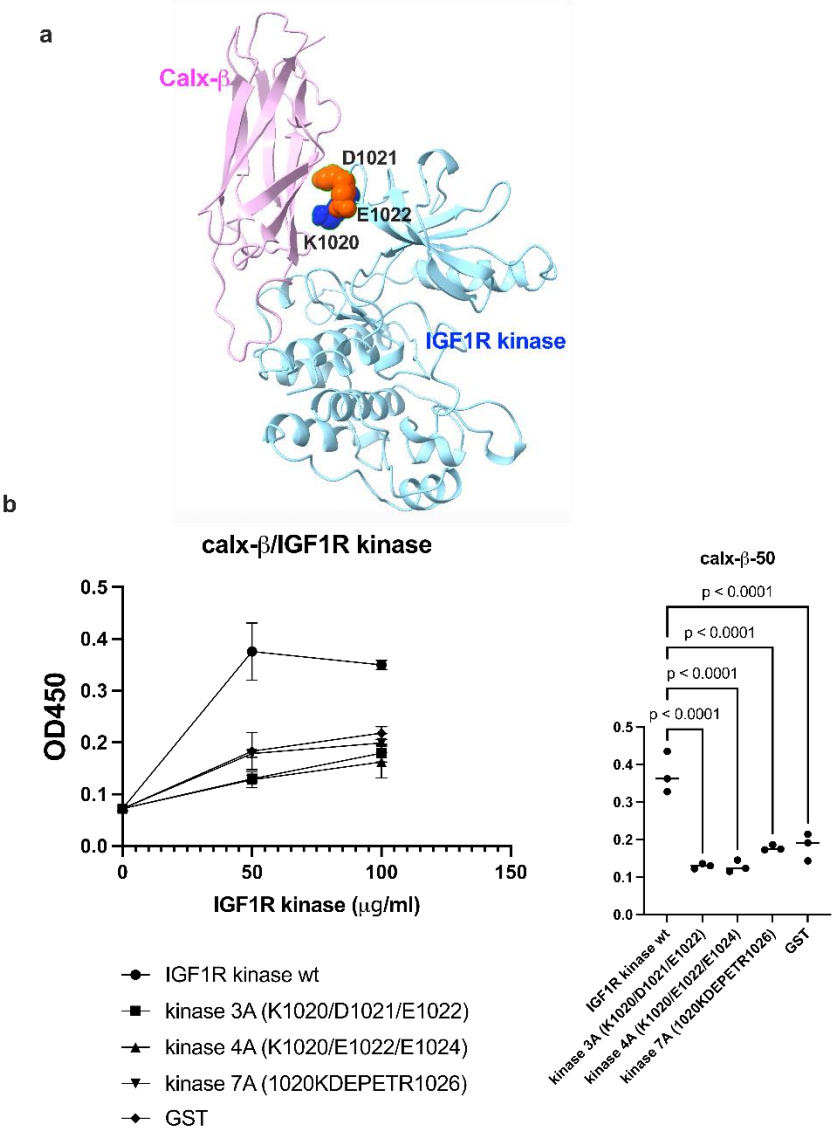

Figure 2. Point mutations in the predicted calx-β binding interface of the IGF1R kinase suppress calx-β binding to IGF1R. (a) We introduced point mutations in the predicted calx-β binding interface of IGF1R kinase. Positions of amino acid residues of the 3A mutant. (b) Effect of point mutations on calx-β binding to IGF1R

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kinase in ELISA. Statistical analysis was performed at 50 μg/mL. Data is shown as means +/- SD of triplicate experiments.

Soluble Calx-β with Tat-Cell-Permeating Peptide (Tat-Calx-β) Markedly Enhances Cell Survival of Keratinocytes (HaCAT) Under Anchorage-Independent and Dependent Conditions

We hypothesized that the calx-β binding to IGF1R kinase is involved in IGF1R kinase activation upon IGF1 binding to $\alpha 6\beta 4$, and, if this is the case, isolated calx-β domain alone will induce enhanced cell survival without IGF1 binding to $\alpha 6\beta 4$. To test this hypothesis, we generated soluble isolated calx-β domain with Tat-cell-permeating peptide (Tat-calx-β). We found that Tat-calx-β in the culture medium markedly enhanced survival of HACAT human keratinocytes (up to 50 μg/mL) in serum-free 2D culture (16 hrs) (Figure 3a) and anchorage-independence (Figure 3b) in dose-dependent manner. We found that much lower Tat-calx-β concentrations in the medium (below 1 μg/mL) was sufficient to enhance cell survival of HaCAT cells in serum-free medium in anchorage-dependent and anchorage-independent (in polyHEMA-coated wells) conditions (Figure 3c). KD for Tat-calx-β was estimated as 0.029 μg/mL (1.4 nM). These findings suggest that calx-β binding to IGF1R kinase is sufficient in inducing survival and proliferation. These findings suggest that IGF1 binding to $\alpha 6\beta 4$ effectively activate calx-β domain, and the isolated Tat-calx-β is fully activated. We found that Tat-calx-β did not enhance survival of breast cancer cell lines (MCF7 and MDA-MB231 cells) in 2D culture in serum-free conditions (Figure 3d). IGF1R kinase is not activated in non-transformed cells but Tat-calx-β binding to the kinase domain activates IGF1R kinase. This is consistent with the idea that IGF1R kinase has already been activated in cancer cells. Heat treatment (80 °C 10 min) of calx-β domain abrogated binding to IGF1R kinase, suggesting that proper folding of calx-β domain is required for binding to IGF1R kinase (Figure 3e).

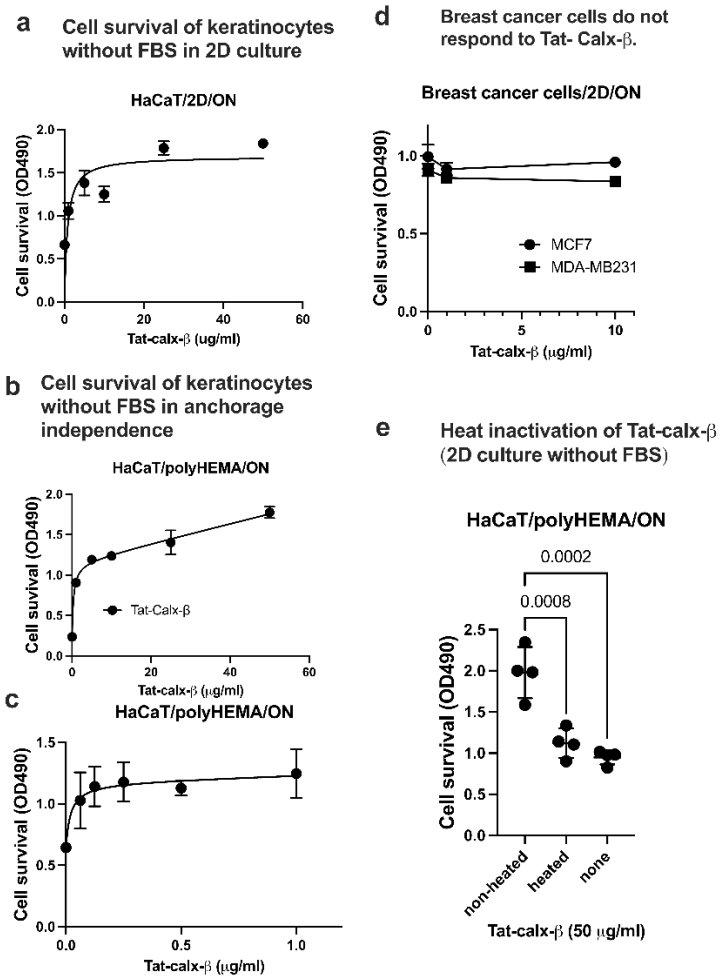

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Figure 3. Tat-calx-β markedly enhances survival of human keratinocytes in a dose-dependent manner in serum-free conditions. Human keratinocytes (HACAT cells) were cultured in DMEM/10% FBS and transferred to 96-well regular serum-free 2D culture plate (a, d and e) or polyHEMA-coated wells (anchorage-independent) (b and c). Cells were cultured in DMEM with no FBS for 16 hrs with Tat-calx-β (1-50 μg/mL). Tat-calx-β potently enhanced cell survival (a and b). (c) Tat-calx-β markedly enhances survival of HaCaT cells below 1 μg/mL in anchorage-independent conditions. (d) Breast cancer cells did not respond to Tat-calx-β, suggesting that the kinases are already activated. (e) Heat denatured Tat-calx-β is inactive. Heat denatured (80 C 10 min) inactivate Tat-calx-β. Cell survival was measured using MTS assays. Data is shown as means +/- SD of triplicate experiments.

We unexpectedly found that Tat-calx-β enhances cell survival of embryonic NIH3T3 fibroblast and C2C12 myoblasts in anchorage-independence, although these cells do not express $\alpha 6\beta 4$ (Figure 4a, 4b). These findings suggest that the isolated Tat-calx-β domain is activated and is able to bind and activate IGF1R kinase without IGF1 binding to $\alpha 6\beta 4$ or without $\alpha 6\beta 4$ expression.

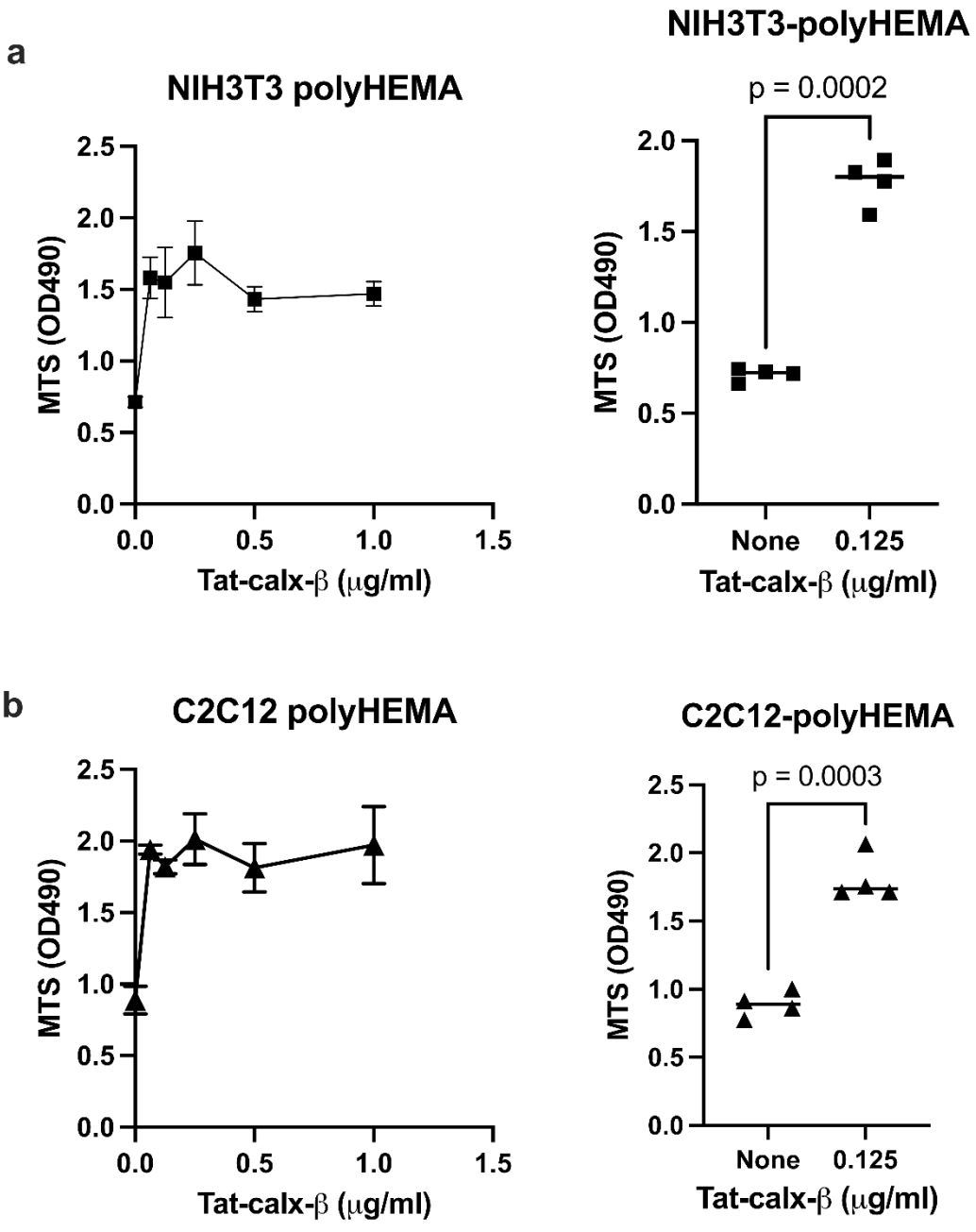

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Figure 4. Tat-calx-β markedly enhances survival of NIH3T3 embryonic fibroblasts and C2C12 myoblasts in a dose-dependent manner in serum-free conditions. Mouse NIH3T3 and C2C12 cells were cultured in DMEM/10% FBS and transferred to 96-well regular serum-free 2D culture plate or polyHEMA-coated wells (anchorage-independent) (b and c). Cells were cultured in DMEM with no FBS for 16 hrs with Tat-calx-β (0-1 μg/mL). Tat-calx-β potently enhanced cell survival (a and b). (c) Tat-calx-β markedly enhances cell survival below 1 μg/mL in anchorage-independent conditions. Cell survival was measured using MTS assays. Data is shown as means +/- SD of triplicate experiments.

Genetic Mutations in Junctional Epidermolysis Bullosa with Pyloric Atresia (JEB-PA) in Calx-β Suppress Calx-β Binding to IGF1R Kinase

JEB-PA is an autosomal recessive inherited rare blistering disorder caused by mutations in α6 or β4. We noticed that several missense JEB-PA mutations in β4 are clustered in the calx-β domain in the binding interface with IGF1R kinase (Figure 5a) (R1014 to W, A1033 to T, S1069 to P, R1072 to Q, R1077 to L, R1078 to G) (These mutations are listed in Uniprot database under P16144). We hypothesized that these mutations suppress calx-β binding to IGF1R kinase and suppress cell survival. We found that these JEB-PA mutations suppressed calx-β binding to the IGF1R kinase domain (Figure 5b–d). To study if calx-β mutants affect cell survival, we generated calx-β with cell-permeating Tat peptide. Several Tat-calx-β with JEB-PA mutants were defective in enhancing survival of keratinocytes (Figure 5e). We propose that JEB-PA calx-β mutants are defective in binding to the IGF1R kinase, resulting in defective IGF1R activation upon ligand binding to α6β4. However, the level of cell survival did not necessarily correlate with that of IGF1R kinase binding. We suspect that this may be because calx-β may bind to multiple kinases other than IGF1R.

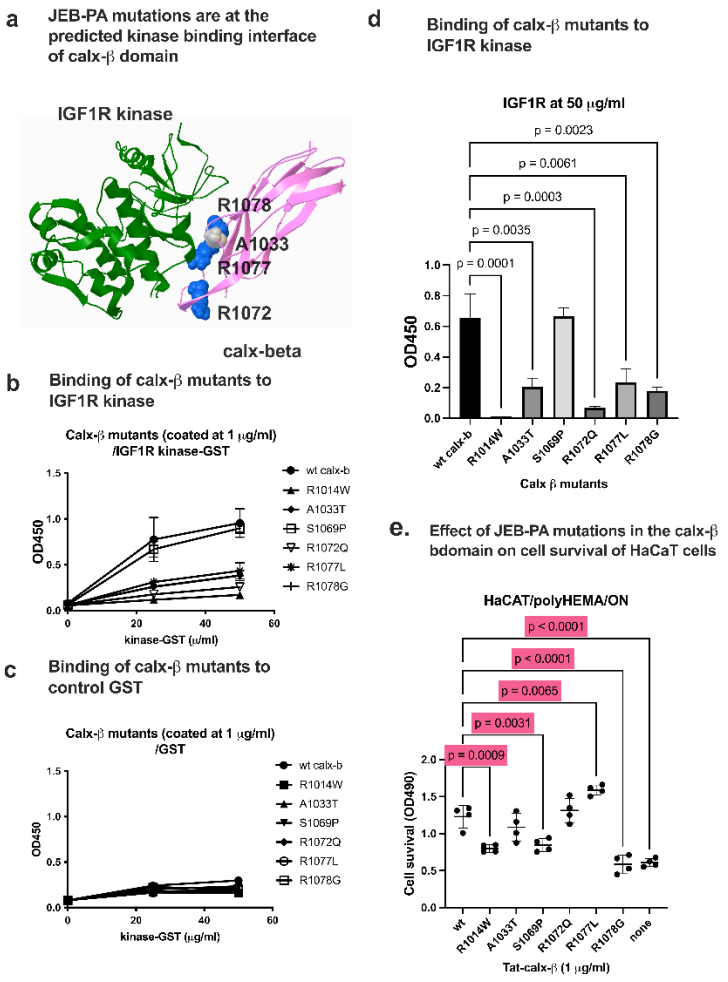

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Figure 5. JEB-PA mutations in the calx-β domain suppress calx-β-binding to IGF1R kinase. (a) JEB-PA mutations are clustered in the IGF1R kinase binding interface of calx-β. (b and c) calx-β binding to IGF1R kinase fused to GST (b) or GST as a negative control (c) was measured as described in Figure 1. Most calx-β mutants are defective in IGF1R kinase binding. (d) calx-β mutants at 50 μg/mL binding to IGF1R kinase. (e) Cell survival of HaCaT cells was measured in MTS assays. Tat-calx-β mutants were incubated with HaCaT cells in anchorage-independence as described in Figure 2c. Multiple JEB-PA mutants of calx-β are defective in enhancing survival of keratinocytes. Data is shown as means +/- SD of triplicate experiments.

Discussion

The present study shows that IGF1 binding to α6β4 effectively triggers the binding of calx-β domain to the IGF1R kinase domain in the α6β4-IGF1-IGF1R ternary complex. Mutation of the predicted the calx-β binding site in IGF1R kinase, and the predicted IGF1R-binding site in calx-β, inhibited the calx-β/IGF1R kinase interaction, suggesting that the docking simulation is correct. To our knowledge this is the first evidence that calx-β of β4 and IGF1R kinase interact.

Notably, the isolated Tat-calx-β domain markedly enhanced cell survival of several non-transformed cells, including keratinocytes, in anchorage independence and without FBS or IGF1. These findings suggest that the calx-β domain binding to IGF1R kinase is sufficient for activating IGF1R kinase and enhancing cell survival. Interestingly, this pathway bypasses IGF1 binding to IGF1R or α6β4. However, Tat-calx-β did not affect survival of cancer cells, suggesting that IGF1R may be constitutively activated in cancer cells. These findings also suggest that IGF1R kinase may be activated by Tat-calx-β without IGF1 binding to IGF1R ectodomain. Furthermore, it is interesting that Tat-calx-β enhances survival of cells that do not express α6β4, suggesting that calx-β binding to IGF1R kinase may be sufficient for cell survival.

It has been proposed that IGF1 binding to IGF1R (ectodomain) induces conformational changes in IGF1R and thereby activates the kinase domain inside the cells [22]. Integrins are not involved in this model. This process can be blocked by the IGF1 R36E/R37E mutant, although the mutant can bind to IGF1R at a comparable level with that of wild type IGF1 [9]. We propose that IGF1 binding to α6β4 triggers calx-β binding to IGF1R kinase and activates IGF1R kinase. It would be critical for IGF1R to tightly associate with α6β4 to induce calx-β binding to IGF1R kinase.

The present study suggests that JEB-PA mutations in calx-β domain affected calx-β binding to IGF1R kinase or cell survival of keratinocytes, suggesting that these mutations may be involved in the pathogenesis of JEB-PA. Since Tat-calx-β enhances survival of keratinocytes and other cell types, Tat-calx-β may be potentially useful to block cell death in blistering diseases like JEB-PA, or in degenerative diseases (e.g., Alzheimer' diseases). It is possible that calx-β may bind to multiple kinases, and the effect of individual JEB-PA mutation may be different among different kinases. Therefore, it is imperative to study the binding specificity of calx-β and the effect of individual JEB-PA mutations on multiple kinases in future studies.

We previously showed that IGF1 binds to integrin αvβ3 and induces αvβ3-IGF1-IGF1R ternary complex [9]. The GF1 mutant defective in αvβ3 binding (R36E/R37E) is defective in signaling and acts as an antagonist, although the mutant binds to IGF1R [23]. In our parallel studies, we discovered that IGF1 binding to αvβ3 triggers the binding of Gα13 to the β3 cytoplasmic domain through the EEE motif. Then, Gα13 binds to the non-catalytic site of IGF1R kinase (the allosteric site) [11]. The β4 tail has no EEE motif. Notably, both Gα13 and calx-β bind to the allosteric site of IGF1R kinase. These findings suggest that Gα13 and calx-β binding to the allosteric site of IGF1R kinase may be a final common pathway in IGF1 signaling leading to IGF1R activation.

Conclusion

The calx-β domain is predicted to bind to IGF1R kinase. Notably, the predicted calx-β binding site to the kinase domain is distinct from the active site of IGF1R kinase and overlaps with Gα13 binding site (the allosteric site). The isolated IGF1R kinase domain directly bound to calx-β in ELISA.

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Point mutations of calx-β/IGF1R interaction suppressed the interaction, suggesting that the docking model is correct. Furthermore, the isolated calx-β domain with cell-penetrating peptide (Tat-calx-β) markedly enhanced survival of keratinocytes in serum-free, and anchorage-independent conditions, suggesting that calx-β binding to IGF1R is sufficient to induce cell survival and proliferation through allosteric activation, and that Tat-calx-β has therapeutic potential for diseases (e.g., degenerative diseases). We propose that IGF1 binding to α6β4 induces calx-β binding to IGF1R kinase and allosterically activates the kinase and subsequently enhances cell survival. Several known missense mutations in the calx-β domain in JEB-PA patients suppressed calx-β binding to IGF1R kinase, suggesting that defective outside-in signaling through α6β4 is related to the pathogenesis of JEB-PA. The present findings suggest a potential model, in which IGF1 binding to α6β4 induces calx-β binding to IGF1R kinase, and then induces IGF1R kinase activation in an allosteric mechanism. There is, however, limitation of the study. The interaction between IGF1R kinase and calx-β domain is supported primarily by in silico modeling and in vitro biochemical assays, and a direct validation of the protein-protein interaction in cells is still required to confirm the proposed model in future studies.

Materials and methods

Synthesis of the calx-β domain. A cDNA fragment encoding the calx-β domain (residues 854–1183)
[VNITIIKEQARDVVSFEQPEFSVSRGDQVARIPVIRRVLDGGKSQVSYRTQDGTAQGNRDYIPVEG ELLFQPGEAWKELQVKLLELQEVDSLLRGRQVRRFHVQLS] was synthesized and subcloned into the Bam HI/Eco RI site of pET28a vector. Protein was synthesized in E. coli BL21 as insoluble inclusion body and purified by Ni/NTA affinity chromatography under denaturing conditions and refolded as described [24]. Tat cell-penetrating peptide (GRKKRRQRRRPP) was inserted at the N-terminus of calx-β domain by inserting the cDNA fragment encoding Tat at the Bam HI site of pET28a vector.

ELISA-type binding assay. Wells of 96 well microtiter plate were incubated with recombinant calx-β (1 μg/mL) in PBS for 1h at room temperature and the remaining binding sites were blocked by incubating with 0.1% heat-treated BSA (80 °C for 10 min). Wells were incubated with the IGF1R kinase domain (fused to GST) in PBS for 1 h at room temperature. Control GST was used as a negative control. After washing the wells, bound IGF1R kinase was quantified using HRP-conjugated anti-GST and peroxidase substrate.

ELISA-type binding assay. Wells of 96 well microtiter plate were incubated with recombinant calx-β (1 μg/mL) in PBS for 1h at room temperature and the remaining binding sites were blocked by incubating with 0.1% heat-treated BSA (80 °C for 10 min). Well were incubated with the IGF1R kinase domain (fused to GST) in PBS for 1 h at room temperature. Control GST was used as a negative control. After washing the wells, bound IGF1R kinase was quantified using HRP-conjugated anti-GST and peroxidase substrate.

Cell survival. Cells were cultured in DMEM/10% FBS and transferred to 96-well regular serum-free 2D culture plate or polyHEMA-coated wells (anchorage-independent). PolyHEMA-coated wells were prepared according to [23,25]. Cells were cultured in DMEM with no FBS for 16 hrs with Tat-calx-β (1-50 μg/mL). Cell survival was measured using MTS assays.

Site-directed mutagenesis was per- formed using the QuickChange method [26].

Docking simulation. Docking simulation between the C-terminal region of calx-β and IGF1R kinase (3QQU.pdb) was performed as described above [27]. The calx-β model based on 3fq4.pdb was generated by Swissmodel. The ligand was presently compiled to a maximum size of 3072 atoms. Atomic solvation parameters and fractional volumes were assigned to the protein atoms by using the AddSol utility, and grid maps were calculated by using the AutoGrid utility in AutoDock 3.05. A grid map with 127 × 127 × 127 points and a grid point spacing of 0.603 Å included the headpiece of αvβ3. Kollman 'united-atom' charges were used. AutoDock 3.05 uses a Lamarckian genetic algorithm (LGA) that couples a typical Darwinian genetic algorithm for global searching with the Solis and Wets algorithm for local searching. The LGA parameters were defined as follows: the initial

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population of random individuals had a size of 50 individuals; each docking was terminated with a maximum number of 1 × 10^6 energy evaluations or a maximum number of 27,000 generations, whichever came first; and mutation and crossover rates were set at 0.02 and 0.80, respectively. An elitism value of 1 was applied, which ensured that the top-ranked individual in the population always survived into the next generation. A maximum of 300 iterations per local search were used. The probability of performing a local search on an individual was 0.06, whereas the maximum number of consecutive successes or failures before doubling or halving the search step size was 4.

Statistical Analysis. We used Prism 10 (Graphpad Software, Boston, MA, USA) to test treatment differences using ANOVA and Tukey multiple comparison tests to control the global type I error.

Table 1. Amino acid residues predicted to be involved in calx-β/IGF1R kinase interaction.

calx-β (-18.18 kcal/mol)	IGF1R kinase (3QQU.pdb)
Pro997, Glu998, Arg1027, Gln1029, Asp1030, Gly1031, Thr1032, Ala1033, Gln1034, Gly1035, Asn1036, Arg1037, Leu1064, Glu1066, Val1067, Asp1068, Ser1069, Leu1070, Leu1071, Arg1074, Gln1075, Val1076, Arg1077, Arg1078, His1080, Val1081, Gln1082, Gln1094, Pro1095, His1096, Ser1097, Thr1098, Thr1099	Lys1020, Asp1021, Glu1022, Pro1023, Glu1024, Arg1026, His1057, Thr1080, Arg1081, Leu1091, Arg1092, Pro1093, Pro1104, Ser1105, Lys1108, Gln1111, Ala1141, Glu1142, Asp1143, Phe1144, Thr1145, Glu1268, Pro1269, Gly1170, Phe1271, Glu1273, Val1274

Amino acid residues within 0.6 nm between calx-β and IGF1R kinase were selected using Pdb Viewer (version 4.1).

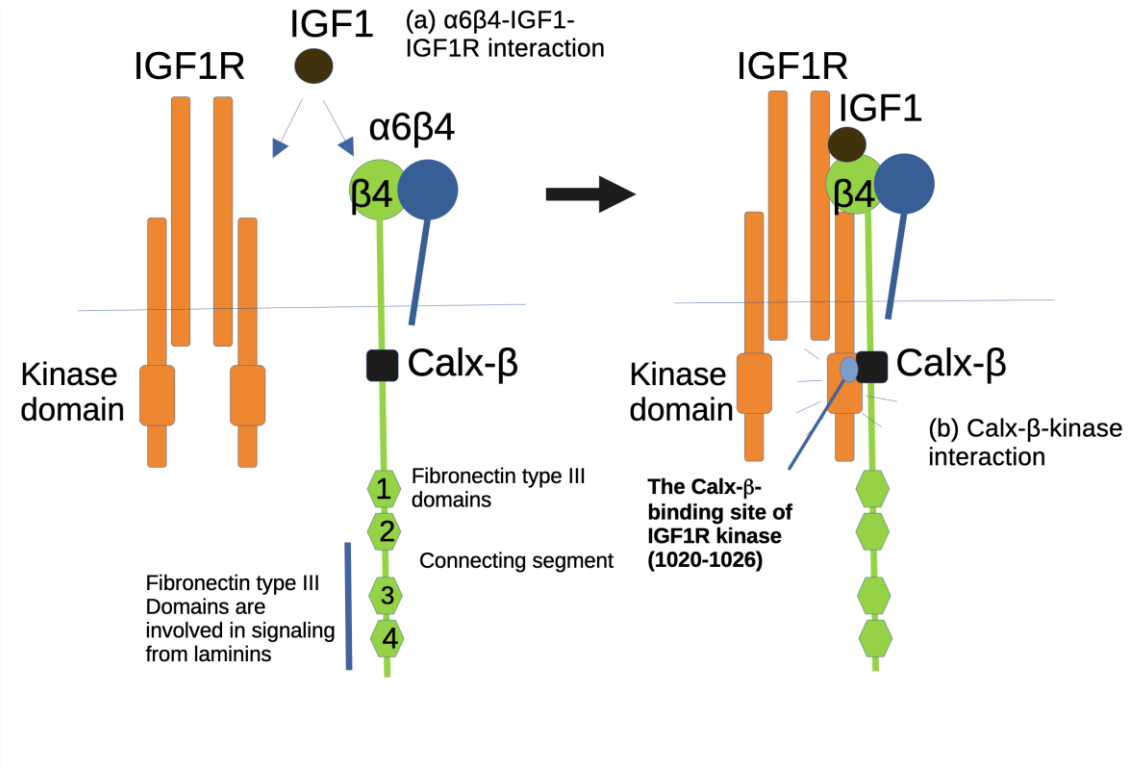

Figure 6. A model of IGF1 signaling through integrin α6β4. Integrin α6β4 is critically involved in IGF1 signaling. IGF1 directly binds to α6β4 and induces α6β4-IGF1-IGF1R ternary complex on the cell surface. The IGF1 mutant defective in integrin binding (the R36E/R37E mutant) is defective in IGF1 signaling and acts as an antagonist, although this mutant still binds to IGF1R. Deletion of β4 tail markedly reduced cell viability and proliferation, suggesting that β4 tail is critically involved in IGF1 signaling. The calx-β domain is predicted to bind to IGF1R kinase in docking simulation. Indeed IGF1R kinase binds to calx-β in ELISA type binding assays. Mutations of IGF1R kinase-binding site in calx-β inhibited IGF1 signaling, suggesting that calx-β binding to IGF1R kinase is critical for IGF1 signaling. Furthermore, isolated calx-β with cell-permeating peptide (Tat) markedly enhance

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cell survival, suggesting that calx-β is sufficient for enhancing cell survival. Notably, the calx-β-binding site in IGF1R kinase (residues 1020-1026) overlaps with that of Gα13-binding site (non-catalytic, allosteric site) (a), suggesting that Gα13 and calx-β-binding to IGF1R kinase is a final common pathway in IGf1 signaling. JEB-PA mutations are clustered in the IGF1R kinase-binding site in the calx-β domain, suggesting that defective IGF1R kinase-calx-β binding may be part of the pathogenesis of JEB-PA.

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