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RIFLE: A Novel Ring Zinc Finger-Leucine-Rich Repeat Containing Protein, Regulates Select Cell Adhesion Molecules in PC12 Cells

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Abstract Cell adhesion molecules play a critical role in cell contacts, whether cell–cell or cell–matrix, and are regulated by multiple signaling pathways. In this report, we identify a novel ring zinc finger-leucine-rich repeat containing protein (RIFLE) and show that RIFLE, expressed in PC12 cells, enhances the Serine (Ser)21/9 phosphorylation of glycogen synthase kinase-3α/β (GSK-3α/β) resulting in the inhibition of GSK-3 kinase activity and increase of β-catenin levels. RIFLE expression also is associated with elevated E-cadherin protein levels but not N-cadherin. The regulation of these cell adhesion-associated molecules by RIFLE is accompanied by a significant increase in cell–cell and cell–matrix adhesion. Moreover, increase in cell–cell adhesion but not cell–matrix adhesion by RIFLE can be mimicked by selective inhibition of GSK-3. Our results suggest that RIFLE represents a novel signaling protein that mediates components of the Wnt/wingless signaling pathway and cell adhesion in PC12 cells. J. Cell. Biochem. 90: 1224–1241, 2003.

Key words: ring zinc finger; leucine-rich repeats; cell adhesion; GSK-3β; β-catenin; kinase activity

The family of leucine-rich repeat (LRR) proteins constitutes a large number of proteins that has been recently divided into six subfamilies typified by distinct lengths (20–29 residues) and consensus sequences [Kajava, 1998]. Members of this family are known to participate in a variety of biological processes. For instance, the LRR is a structural module involved in molecular recognition processes such as cell adhesion, signal transduction, DNA repair, and RNA processing [Iozzo, 1999]. Like the LRR, the ring zinc finger (RZF) motif has also been found in a variety of eukaryotic proteins of diverse evolutionary origin that are involved in various cellular processes such as oncogenesis, development, signal transduction, and apoptosis [Iuchi, 2001; Kroncke, 2001; Laity et al., 2001; Pabo et al., 2001]. RZF is known to play a role in mediating protein–DNA binding and protein–protein interaction, both of which are characteristic features of a transcriptional factor.

Cell adhesion to neighboring cells or to the surrounding extracellular matrix (ECM) is a vital function necessary for cell survival, migration, proliferation, and differentiation during CNS development [Adams and Watt, 1993; Sastry and Horwitz, 1996; Hynes, 1999; Benson et al., 2000]. Gene regulation in response to signals from the environment is usually required for cell adhesion interactions. This process involves signal-dependent-transcriptional regulation and subsequent modulation of adhesion-related molecules. The cadherin–catenin complex is well known for mediating homotypic calcium-dependent cell adhesion and recognition in diverse tissue types and is regulated by components of the Wnt/wingless signaling pathway, specifically glycogen synthase kinase-3 (GSK-3)/β-catenin [Papkoff and Aikawa, 1998;

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Dierick and Bejsovec, 1999; Novak and Dedhar, 1999; Waltzer and Bienz, 1999]. GSK-3α and β are closely related serine/threonine kinases that act as inhibitory components of Wnt/wingless signaling during embryonic development. Inhibition of GSK-3 kinase activity by dominant negative GSK-3 mutants leads to activation of the Wnt signaling pathway in *Dictyostelium*, *Drosophila*, *Xenopus*, and mammalian cells [Cadigan and Nusse, 1997; Dale, 1998; Wodarz and Nusse, 1998]. In PC12 cells, both lithium and dominant negative GSK-3 mutants can mimic Wnt signaling [Stambolic et al., 1996; Bournat et al., 2000]. Recent studies, along with the elucidation of the GSK-3β three-dimensional structure, have significantly enhanced our understanding of the molecular basis for β-catenin regulation by GSK-3. Activation of Wnt signaling results in inhibition of GSK-3 kinase activity by disrupting a multiprotein complex comprising GSK-3 and its substrates such as β-catenin. GSK-3 is then unable to tag β-catenin by phosphorylation for degradation which in turn leads to the stability of β-catenin [Cohen and Frame, 2001; Woodgett, 2001]. Moreover, β-catenin is able to associate with the C-terminal cytoplasmic domain of cadherins to link extracellular adhesion with actin cytoskeleton [Nagafuchi and Takeichi, 1988; Ozawa et al., 1990; Knudsen et al., 1995; Rimm et al., 1995] and/or is translocated to the nucleus where it forms a complex with Tcf/Lef-1 transcription factors [Kikuchi, 2000].

In this study, we identify a novel ring zinc finger-leucine-rich repeat containing protein, RIFLE, by using Incyte database screening to a conserved RZF region and reveal that RIFLE protein contains three other putative functional motifs in addition to the RZF domain: LRRs, a leucine zipper domain and a nuclear localization signal (NLS) domain. Although largely divergent in sequence, RIFLE shares similar structural motifs with other RZF–leucine zipper containing protein such as c-RZF. C-RZF is a putative transcriptional factor that was identified by subtractive hybridization from gene expression that occurs when the substrate adhesion molecules cytotactin/tenascin bind to neurons [Tranque et al., 1996]. This prompted us to investigate whether RIFLE may play a role in regulating cell adhesion molecules and cell adhesion in the PC12 pheochromocytoma cells, a neural crest-derived tumor cell line with both neuronal and epithelial characteristics [Greene and Tischler, 1976; Franke et al., 1986].

MATERIALS AND METHODS

Cloning of RIFLE cDNA and Construction of RIFLE Expression Vector

A partial RIFLE coding cDNA fragment was identified by searching the Incyte cDNA database using a conserved RZF region of a neuronal apoptosis inhibitory protein (*NAIP*) gene as bait. A 3.14 kb RIFLE cDNA containing an open reading frame was identified by screening the human brain cDNA library with standard methods. The 3.14 kb cDNA was cut out of *pBluescript* vector with Spe I, and then ligated in a *pBI–EGFP* expression vector using an EcoR V site to form the *pBI–EGFP–RIFLE* expression vector. The pBI–EGFP expression vector is a bi-directional doxycycline regulated plasmid that expresses EGFP simultaneously with RIFLE. In addition, a DNA fragment coding the hemagglutinin (HA) epitope was cloned at the 3′-end of the RIFLE cDNA and placed into a pcDNA3.1(+) vector to form the *pcDNA3.1/RIFLE–HA* expression vector.

Cell Culture

All cells were cultured at 37°C and 5% CO_2. Naive PC12 cells were cultured in DMEM (GIBCO, Gaitherburg, MD) supplemented with 10% horse serum (GIBCO), 5% fetal bovine serum (Clontech, Palo Alto, CA) and 1% Pen/Strep. *pcDNA3.1/RIFLE–HA* tranfected cells and tet-off system parental PC12 cells (Clontech) were maintained in naive PC12 medium containing Geneticin (GIBCO, 200 μg/ml). *pBI–EGFP–RIFLE* tet-off transfected clones were cultured in parental cell medium containing hygromycin (Clontech, 200 μg/ml) but without Doxycycline for continual on expression.

Transfections

Twenty-four hours prior to transfection, 1×10^6 parental cells were seeded on individual collagen IV 60-mm plates (Becton Dickinson, Franklin Lakes, NJ). Cells were cultured to 80% confluence. The transfection of the *pBI–EGFP–RIFLE* construct into parental PC12 tet-off cells was performed by using lipofectamine-plus following the manufacture's instruction (Gibco/BRL). After selecting with hygromycin (200 μg/ml) for 2 weeks, 20 individual clones were picked, expanded, and frozen down for further

analysis. For maintenance of the RIFLE transfected cells, the concentration of G418 and hygromycin were kept the same as in the selection (200 μg/ml) process. Due to leakiness of the vector, *pBI–EGFP–RIFLE* transfected cells were maintained under continual expression conditions, that is without the presence of Doxycycline.

pcDNA3.1/RIFLE–HA was transfected into naive PC12 cells using the Lipofectamine method as described above. Following a 2-week selection with G418 (500 μg/ml), *pcDNA3.1/RIFLE–HA* PC12 colonies were pooled and maintained in standard PC12 culture medium containing 200 μg/ml G418. A *pBI–EGFP* vector and *GSK-3β KK* (kinase dead) construct was introduced into PC12 tet-off cells (Clontech) using electroporation as previously described [Melemed et al., 1997]. Cells were selected in media supplemented with 200 μg/ml G418 and 100 μg/ml hygromycin. Individual clones containing the *GSK-3β KK* construct were picked, characterized, and maintained under selection media conditions and, as with *pBI–EGFP–RIFLE* clones, without Doxycycline for continual on expression.

Northern Blot Analysis

Total RNA was isolated from RIFLE and vector control cells using Trizol (GIBCO) following manufacture's instruction and subjected to Northern blot analysis as described previously [Li et al., 1994]. Full length RIFLE cDNA was used as a Northern probe. The Clontech multiple tissue northern (MTN) blot was also used in this analysis.

Cell–Cell Adhesion Assays

PC12 cells stably transfected with *pcDNA3.1/RIFLE–HA* or *GSK-3β KK* (kinase dead) constructs were analyzed for cell–cell adhesion by examining defined cell clusters following the method of Rothlein's [Rothlein and Springer, 1986] with modification. Briefly, PC12 cells were cultured in complete medium for at least 24 h. The cells were washed twice with 1× PBS and re-suspended in tissue culture medium without serum to a concentration of 2×10^6 cells/ml. Five microliters of calcein AM stock solution (Molecular Probes, Pittsburgh, PA) was added into 1 ml of cell suspension to achieve a final concentration of 5 μM. The mixture was incubated at 37°C for 30 min. Cells were washed twice with pre-warmed (37°C) culture medium and re-suspended in culture medium at 2×10^6 cells/ml. One hundred microliters of the calcein-labeled cell suspension (2×10^6 cells/ml) was combined with either 1 mM Ca^{++} or 1 mM EGTA and was transferred to a glass slide. Cells were incubated on glass slides at 37°C. The formation of aggregated cell clusters ($n \geq 5$ cells) was examined after 1–2 h using phase contrast microscopy. For quantitative aggregation assay, cells were allowed to settle spontaneously and the degree of aggregation was scored at indicated time. The number of free cells and aggregated cells were quantified. Percent aggregation was determined by the following equation: percent aggregation = 100 × (number of aggregated cells forming clusters/total number of cells). Total number of cells equals the number aggregated cells forming clusters plus the number of free cells. Cell–cell adhesion antibody neutralization studies were done using the same procedure adding either E-cadherin antibody (DECMA-1, Sigma) [Zantek et al., 1999] or control antibody IgG (Rabbit IgG, KPL, Gaithersburg, MD) together with calcein. The final concentration for both antibodies was 44 μg/ml.

Cell–Matrix Adhesion Assays

Cell–matrix adhesion assays were performed on CytoMatrix cell adhesion strips (Chemicon, Temecula, CA) pre-coated with human collagen IV or human fibronectin by the manufacturer with one row of each strip pre-coated with 10% bovine serum albumin (BSA) as a negative control. PC12 cells were sub-cultured 1 day prior to adhesion assay to reach 90–100% confluence. The cells were detached from the flasks using a cell scraper and a single cell suspension of 5×10^6 cells per ml was prepared by pipetting the cells up and down about 20 times followed by counting using a Coulter particle counter (Coulter Cooperation, Miami, FL). During the preparation of the cell suspension, the CytoMatrix cell adhesion strip wells were re-hydrated with 1× PBS for 15 min at room temperature. One hundred microliters of the cell suspension containing 5×10^5 cells was plated in each well of the CytoMatrix cell adhesion strips and were allowed to attach for 1 h at 37°C in a humidified 5% CO_2 atmosphere. The non-adherent cells were removed by gently washing the cells with PBS containing Ca^{2+} and Mg^{2+} and then the adherent fraction was quantified using crystal violet staining. Briefly, after washing the wells,

100 μl of 0.2 % crystal violet in 10% ethanol was added to each well and the plates were incubated at room temperature for 5 min. The staining solution was then removed and the wells were gently washed 3–5 times with PBS to remove the excess stain. One hundred microliters of solubilization buffer (a 50/50 mixture of 0.1 m NaH_2PO_4, pH 4.5 and 50% ethanol) was then added to each well. Following solubilization, the absorbance of each well was measured in a microplate reader (Labsystems, Franklin, MA) at 540 nm with a reference wavelength of 690 nm. The OD_{540} values of each RIFLE clone and vector control were then subject to statistical analysis using the Student's *t*-test.

Western Blot Analysis

At time of study, for analyzing cell membrane proteins, cells were harvested via cell scraper (Fisher, Pittsburgh, PA), washed with PBS and then dounced homogenized (Kontes dounce) in lysis buffer (10 mM K_2HPO_4 pH 7.2, 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 1 mM Na_3VO_4, 2 mM DTT, 1% Triton X-100, 1 μM Microcystin, COMPLETE protease inhibitor tablet (Roche, Indianapolis, IN)) and incubated on ice for 30 min. The total lysate was clarified by microcentrifuging at 14,000 rpm for 30 min at 4°C. Total protein concentration of the supernatants was determined using the BCA Protein Assay (Pierce, Rockford, IL). For analyzing intracellular proteins, cells were detached from flask or plate with 0.25% trypsin–EDTA, rinsed once with 1× PBS, then flash frozen in liquid nitrogen, and stored at −80°C for future analysis. Whole cell lysate was prepared by re-suspending cell pellets in lysis buffer (see above), then following same procedure as scraping harvested cells. Thirty micrograms of total protein from each sample was then fractionated on a 10% NuPAGE Bis-Tris gel and transferred onto a 0.2 μm nitrocellulose membrane (Novex, San Diego, CA) using a Hoefer Transblotter (Semiphor, San Francisco, CA). The membrane was washed twice for 5 min each in TST (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 7.5), blocked with 5% BSA–TST for 1 h at room temperature on a shaker platform (Lab-Line) and then incubated with respective primary antibody (β-catenin, E-cadherin, α-actinin, and GSK-3 protein antibodies were purchased from Upstate Biotechnology (Lake Placid, NY); phospho-GSK-3 and phosphor-PKC antibodies were purchased from Cell Signaling Technology (Boston, MA); intercellular cell adhesion molecule (ICAM), cadherin-5, and vascular cell adhesion molecule (VCAM) antibodies were purchased from Santa Cruz (Santa Cruz, CA); HA antibody was ordered from Roche (Indianapolis, IN)) overnight at 4°C. The following day, the membrane was washed three times for 5 min each with TST. An appropriate HRP conjugated secondary antibody was diluted 1:2,000 in 5% milk–TST and incubated with the membrane for 1 h shaking at room temperature. Finally, the membrane was washed as above followed by one wash with PBS for 5 min. Respective protein on the membrane was then visualized by Chemiluminescence (Amersham Bioscience, Piscataway, NJ).

GSK-3β Kinase Activity Assay

The GSK-3 kinase activity was measured using a CREB substrate peptide as described [Wang et al., 1994]. Briefly, the kinase reaction occurred in a 50 μl total volume containing 20 mM MOPS pH 7.4, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM NA_3VO_4, 1 mM DTT, 15 mM $MgCl_2$, 100 μM cold ATP, 200 μM CREB peptide (KRREILSRRPpSYR, AnaSpec, San Jose, CA), 10 μl whole cell lysate, and 5 μCi γ-^{33}P-ATP. The reactions were incubated for 30 min at 30°C in a Costar round-96 polypropylene plate. Reactions were then stopped with the addition of 10% H_3PO_4 and transferred to a Millipore MAPH-NOB 96-well phosphocellulose plate. Next, the reactions were incubated at room temperature for 1.5 h, filtered and washed once with 320 μl 0.75% H_3PO_4, and then filtered and washed twice with 160 μl H_3PO_4 at the same concentration using a vacuum manifold (Millipore, Bedford, MA). The filter plate was then placed in a carrier plate and 100 μl of Microscint 20 (Packard, Meriden, CT) was added to each well. The plate was sealed with sealing tape and incubated overnight at room temperature. The following day, the filter plate was read for ^{33}P on Top Count (Packard, Albertville, MN). Finally, CPM was normalized to CPM per μg of total protein.

Statistical Analysis

All statistical analysis for Western blot and cell adhesion studies was completed using the Student's *t*-test with a standard *P* value threshold of ≤ 0.05.

RESULTS

Cloning and Tissue Distribution of RIFLE

We used a conserved RZF region of a *NAIP* gene as bait to search the Incyte database and identified a 1.2 kb cDNA fragment. This 1.2 kb cDNA fragment was subsequently used to screen a human brain cDNA library. Two individual cDNA clones were identified and subjected to further sequence analysis. Both cDNAs with different lengths contained an identical open reading frame that encoded a protein of 702 amino acids with a calculated molecular mass of 80 kDa. The first start codon (methionine) of the longest open reading frame was preceded by two consecutive in-frame stop codons at −15 and −54 bp. The longer cDNA (3,144 bp), comprised of an open coding sequence, a long 5′-flanking region and 3′-untranslated region (Fig. 1A), was named RIFLE and was used in the studies described below. Database searching revealed that RIFLE is a novel protein with no significant homology with any known cDNAs or proteins contained in the database. Protein sequence analysis reveals that RIFLE has a LRR motif (amino acid 59–150), a NLS (amino acids 234–242), a leucine zipper domain (amino acids 528–556), and a RZF motif (amino acids 654–702) (Fig. 1B).

RIFLE is composed of four LRRs, each of which consists of 24 residues, a general characteristic of the LRR family. The consensus sequence of LRR in RIFLE (Fig. 1C,D), however, differs from that of all known six subfamilies. There are two and four amino acid residues following the second and the third leucine, respectively in the LRR consensus sequences of RIFLE instead of the usual one and two amino acids at the same positions in the LRR consensus sequences of the known six subfamilies (Fig. 1D), suggesting that RIFLE might belong to a novel LRR subfamily [Kajava, 1998].

The tissue distribution of RIFLE expression was examined in multiple human tissues and different human brain regions by using Clontech RNA blots. The full length RIFLE cDNA was used as the Northern blot probe. RIFLE was mainly expressed in human brain, heart, skeletal muscle, kidney, and liver (Fig. 1E). Further, RIFLE was expressed in all brain regions examined (Fig. 1F). The molecular size of the RIFLE mRNA as measured by the Northern blots is consistent with the calculated length of the longer clone 3.14 kb, confirming that the longer clone is essentially a full length human RIFLE cDNA.

The non-tagged RIFLE cDNA was cloned into the pBI–EGFP expression vector (pBI–EGFP–RIFLE). RIFLE expression at the mRNA level from three independent colonies stably transfected with *pBI–EGFP–RIFLE* was confirmed by Northern blot analysis (Fig. 2A). Control PC12 cells have no detectable endogenous RIFLE and the three colonies (#10, #14, and #23) express RIFLE mRNAs at similar levels. The expression of RIFLE at the protein level from the pooled population of the PC12 cells transfected with *pcDNA3.1/RIFLE–HA* was measured by Western blot using an anti-HA affinity antibody. Sub-cellular distribution of RIFLE was analyzed in the RIFLE–HA/PC12 cells as previously described [Ahmed et al., 1993] (Fig. 2C). RIFLE is expressed in the cytosol and nuclear fractions, consistent with the presence of NLS and RZF motifs. Both the *pCDNA3.1/RIFLE–HA* and the *pBI–EGFP–RIFLE* cells expressed RIFLE without causing changes in cell viability as assessed by MTT analysis and cell proliferation (data not shown).

Expression of RIFLE Facilitates β-Catenin Protein Accumulation

We further investigated the PC12 cells expressing RIFLE to assess RIFLE's potential role in the regulation of cell adhesion related molecules. We surveyed the expression of several adhesion proteins in response to RIFLE expression in PC12 cells. We found that β-catenin protein, a mammalian homolog of Armadillo, is significantly increased in independent lines of *pBI–EGFP–RIFLE* expressing PC12 cells (Fig. 3A). RIFLE, however, does not change the expression of other adhesion related molecules such as cadherin-5, ICAM-2 (Fig. 3B,C), ICAM-1, VCAM-1, P-cadherin, and E-selectin (data not shown) under the same conditions. The β-catenin protein levels were quantified by densitometry analysis and shown to be statistically elevated in the pBI–EGFP–RIFLE cells as compared to control PC12 cells transfected with vector alone (Fig. 3D). These data suggest that RIFLE selectively regulates β-catenin protein levels. β-Catenin is a key player in the WNT signaling pathway and therefore this data suggest that RIFLE may mimic or augment WNT signaling.

A

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5'-ttccacaggaaataccggatcccagctgatgtagaccctctcaccattacttcatccctgtcatctgatggggtcct
cactgtgaatggaccaaggaaacaggtctctggccctgagcgcaccattcccatcacccgtgaagagaagcctgctgtca
ccgcagcccccaagaaatagatgccctttcttgaattgcatttttaaaacaagaaagtttccccaccagtgaatgaaag
tcttgtgactagtgctgaagccgctcttcttccggaagcggaaacccagtgaggaggctcggaaacgcctggagtaccag

318/1
atg tgt ttg gca aaa gaa gct ggg gca gat gac att ctc gac atc gct gaa tgt gag ctc
M   C   L   A   K   E   A   G   A   D   D   I   L   D   I   A   E   C   E   L
378/21
tca gag att cca ttt gga gct ttt gca aca tgc aaa gtt ctg cag aag aag gtg ctg atc
S   E   I   P   F   G   A   F   A   T   C   K   V   L   Q   K   K   V   L   I
438/41
gtc cac acg aat cac ctc act tcc ctg ctt ccc aaa tcc tgc agc ctc ctg agt ctg gca
V   H   T   N   H   L   T   S   L   L   P   K   S   C   S   L   L   S   L   A
498/61
acc att aag gtt cta gat ctc cac gat aat cag ctg aca gcc ctt cct gac gat ctg ggg
T   I   K   V   L   D   L   H   D   N   Q   L   T   A   L   P   D   D   L   G
558/81
cag ctg act gcc ctc cag gtc tta aac gtg gaa agg aat caa ctg atg cag ctc cca cgt
Q   L   T   A   L   Q   V   L   N   V   E   R   N   Q   L   M   Q   L   P   R

618/101
tcc att ggg aac ctg acc cag ctc cag act ctc aat gtt aaa gac aac aag ctg aag gag
S   I   G   N   L   T   Q   L   Q   T   L   N   V   K   D   N   K   L   K   E
678/121
ctt cca gac acc gtg ggg gag ctt cga agc ctg cgt acc ctc aac atc agt gga aac gag
L   P   D   T   V   G   E   L   R   S   L   R   T   L   N   I   S   G   N   E

738/141
atc cag aga ttg ccg cag atg ctg gct cac gtt cga acc ctg gag atg ctg agc ctt gac
I   Q   R   L   P   Q   M   L   A   H   V   R   T   L   E   M   L   S   L   D

738/161
gcc tcg gcc atg gtc tac ccg ccg cgg gag gtg tgt ggt gcc ggc act acg gcc atc ttg
A   S   A   M   V   Y   P   P   R   E   V   C   G   A   G   T   T   A   I   L
858/181
cag ttc ctc tgc aaa gag tca ggg ctg gaa tac tac ccc cct tct cag tac ttg ctg cca
Q   F   L   C   K   E   S   G   L   E   Y   Y   P   P   S   Q   Y   L   L   P
918/201
att ctg gag caa gat gga atc gag aac tct cgg gac agc cct gat ggg ccc acg gac aga
I   L   E   Q   D   G   I   E   N   S   R   D   S   P   D   G   P   T   D   R
978/221
ttc tca agg gag gag tta gag tgg cag aac agg ttc tca gac tat gag aag agg aag gaa
F   S   R   E   E   L   E   W   Q   N   R   F   S   D   Y   E   K   R   K   E

                                                                    NLS
1038/241
cag aag atg ctg gag aaa ctc gag ttt gaa cgg cgc ctg gaa ctg ggg cag cgg gag cac
Q   K   M   L   E   K   L   E   F   E   R   R   L   E   L   G   Q   R   E   H
1098/261
acc cag ctc ctt cag cag agc agc agc cag aag gat gag atc ctt cag acg gtc aag gag
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Fig. 1. **Panel A**: Nucleotide and deduced amino acid sequence of RIFLE. The deduced amino acid sequence beginning with the initiating methionine is given. Numbering of nucleotide and amino acids is shown at the top. The leucine-rich repeat (LRR) sequences are underlined; the sequences of the nuclear localization signal (NLS), the leucine zipper and ring zinc finger (RZF) are framed. **Panel B**: Schematic structure of RIFLE protein showing the localizations of LRR, NLS, leucine zipper, and RZF motifs. **Panel C**: Alignment of leucine repeat sequences showing the localizations, length, and consensus sequences. **Panel D**: Comparison of the LRR consensus sequences between RIFLE and other LRR containing proteins. Unlike the one derived from the known six subfamilies of LRR proteins, there are two and four amino acid residues following the second and the third leucine, respectively in the LRR consensus of RIFLE, suggesting that RIFLE might belong to a novel LRR subfamily. **Panel E**: Northern blot analysis of human tissue distribution of RIFLE. **Lane 1**: RNA molecular marker; **lanes 2–13**, RNA from whole brain, heart, skeletal muscle, colon, thymus, spleen, kidney, liver, small intestine, placenta, lung, and peripheral blood leukocyte, respectively. **Panel F**: Regional distribution of RIFLE expression in human brain. Shown is RNA blot analysis (1 μg) of mRNA using Clontech prepared human mRNA blot. Lane 1: RNA molecular marker; lanes 2–9 are loaded RNA from cerebellum, cerebral cortex, medulla, spinal cord, occipital pole, frontal lobe, temporal lobe, and putamen, respectively. Both panel E and F are probed using RIFLE full length cDNA.

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T   Q   L   L   Q   Q   S   S   S   Q   K   D   E   I   L   Q   T   V   K   E
1158/281
gag cag tcc cgg ctg gag cag ggc ctg agt gag cac cag cgc cac ctc gac gca gag cgg
E   Q   S   R   L   E   Q   G   L   S   E   H   Q   R   H   L   D   A   E   R


1218/301
cag cgg ctg cag gag cag ctg aag cag acg gaa cag aac att tcc agc cgg atc cag aag
Q   R   L   Q   E   Q   L   K   Q   T   E   Q   N   I   S   S   R   I   Q   K
1278/321
ctg ctg cag gac aat cag aga caa aag aaa agc tcc gag att ttg aaa tcg ctg gaa aat
L   L   Q   D   N   Q   R   Q   K   K   S   S   E   I   L   K   S   L   E   N

1338/341
gaa aga ata aga atg gaa cag ttg atg tcc ata acc cag gag gag act gag agc ctg cgg
E   R   I   R   M   E   Q   L   M   S   I   T   Q   E   E   T   E   S   L   R

1398/361
cga cgt gac gtt gcc tcc gcc atg cag cag atg ctg act gag agc tgt aag aac cgg ctc
R   R   D   V   A   S   A   M   Q   Q   M   L   T   E   S   C   K   N   R   L
1458/381
atc cag atg gcc tac gaa tct cag agg cag aac ttg gtc cag cag gcc tgt tcc agc atg
I   Q   M   A   Y   E   S   Q   R   Q   N   L   V   Q   Q   A   C   S   S   M
1518/401
gcc gaa atg gat gaa cga ttc cag cag att ctg tcg tgg cag caa atg gat cag aac aaa
A   E   M   D   E   R   F   Q   Q   I   L   S   W   Q   Q   M   D   Q   N   K
1578/421
gcc atc agc cag atc ctg cag gag agc gcg atg cag aag gct gcg ttc gag gca ctc cag
A   I   S   Q   I   L   Q   E   S   A   M   Q   K   A   A   F   E   A   L   Q
1638/441
gtg aag aaa gac ctg atg cat cgg cag atc agg agc cag att aag tta ata gaa act gag
V   K   K   D   L   M   H   R   Q   I   R   S   Q   I   K   L   I   E   T   E
1698/461
tta ttg cag ctg aca cag ctg gag tta aag agg aag tcc ctg gac aca gag tca ctc cag
L   L   Q   L   T   Q   L   E   L   K   R   K   S   L   D   T   E   S   L   Q

1758/481
gag atg atc tcg gag cag cgc tgg gcc ctc agc tcc ctg ctc cag cag ctg ctc aaa gag
E   M   I   S   E   Q   R   W   A   L   S   S   L   L   Q   Q   L   L   K   E
1818/501
aag cag cag cga gag gaa gag ctc cgg gaa atc ctg acg gag tta gaa gcc aaa agt gaa
K   Q   Q   R   E   E   E   L   R   E   I   L   T   E   L   E   A   K   S   E
1878/521
acc agg caa gaa aat tac tgg ctg att cag tat caa cgg ctt ttg aac cag aag ccc ttg
T   R   Q   E   N   Y   W   L   I   Q   Y   Q   R   L   L   N   Q   K   P   L
2938/541
tcc ttg aag ctg caa gaa gag ggg atg gag cgc cag ctg gtg gcc ctc ctg gag gag ctg
S   L   K   L   Q   E   E   G   M   E   R   Q   L   V   A   L   L   E   E   L
2998/561                        Leucine Zipper
tcg gct gag cac tac ctg ccc atc ttt gcg cac cac cgc ctc tca ctg gac ctg ctg agc
S   A   E   H   Y   L   P   I   F   A   H   H   R   L   S   L   D   L   L   S

2058/581
caa atg agc cca ggg gac ctg gcc aag gtg ggc gtc tca gaa gct ggc ctg cag cac gag
Q   M   S   P   G   D   L   A   K   V   G   V   S   E   A   G   L   Q   H   E
2118/601
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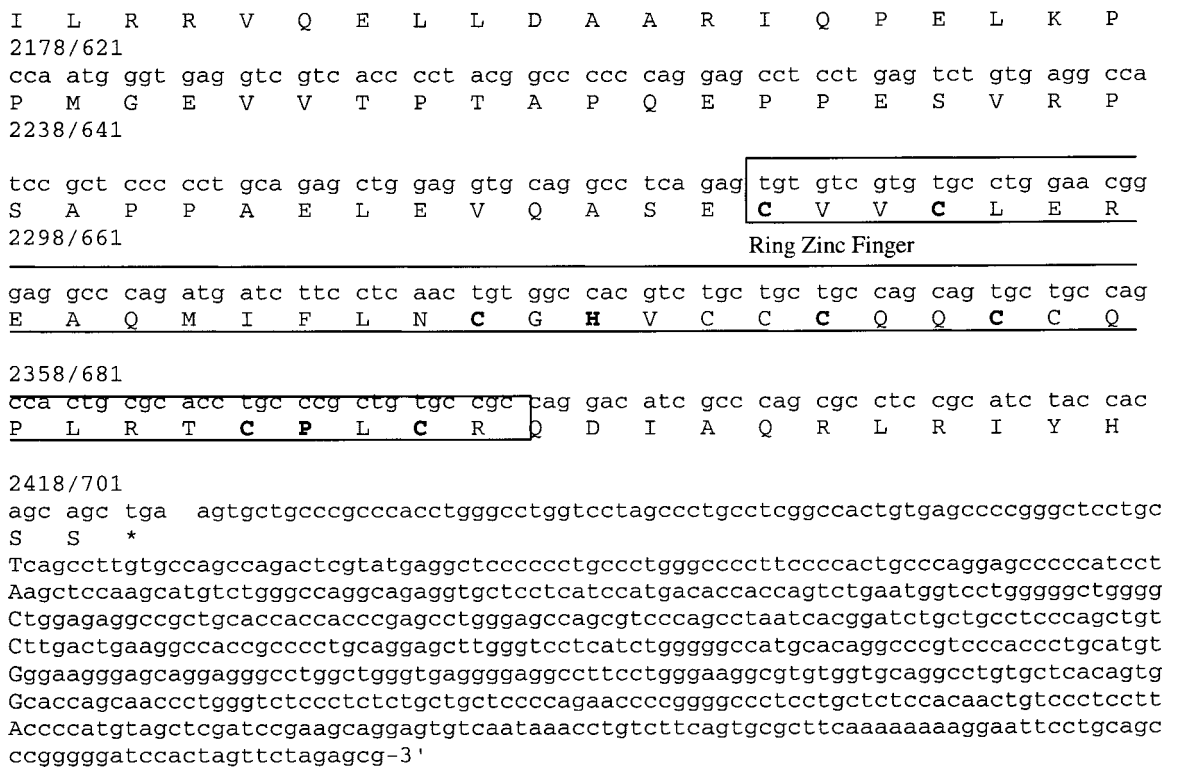
Fig. 1. (*Continued*)



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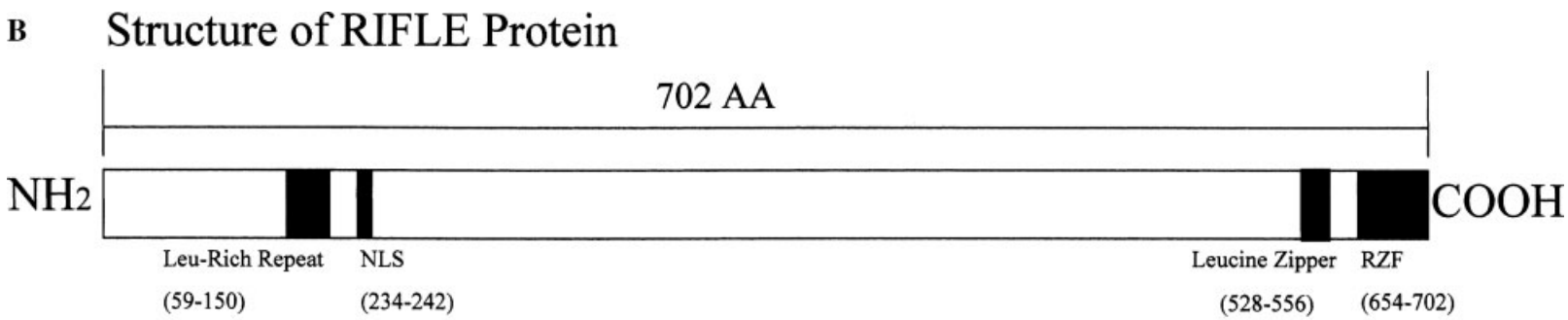
RIFLE Regulates E-Cadherin Expression

β-Catenin is a well-known cell adhesion associated molecule that can physically associate with the C-terminal cytoplasmic domain of cadherins [Huber et al., 1997]. In light of the evidence that β-catenin and cadherin are usually co-regulated, particularly by Wnt/wingless signaling [Porfiri et al., 1997; Stewart et al., 2000], we sought to investigate whether increased levels of β-catenin by RIFLE modulates either E-cadherin or N-cadherin in the RIFLE/PC12 cells by examining cadherin protein levels. While N-cadherin levels did not differ significantly between RIFLE and control cells, a striking change was observed, by Western blotting analysis, in E-cadherin expression in the RIFLE/PC12 cells. RIFLE, as with β-catenin (Fig. 4A,B), elevated the E-cadherin expression levels (Fig. 4C, compare lanes 1 and 2) but did not affect the N-cadherin (Fig. 4D) or α-actinin expression levels (Fig. 4E), suggesting that E-cadherin is selectively co-regulated with β-catenin by RIFLE. The protein levels of RIFLE, β-catenin, E-cadherin, and N-cadherin were quantified by densitometry analysis normalized with α-actinin (Fig. 4F–I) showing that β-catenin (Fig. 4G) and E-cadherin (Fig. 4H) protein levels are significantly elevated in the RIFLE/PC12 cells, respectively as compared to control PC12 cells transfected with vector alone. Our data is consistent with the findings that expression of Wnt-1 in PC12 cells results in increased E-cadherin levels [Bradley et al., 1993; Hollmann et al., 2001].

Expression of RIFLE Regulates Components of WNT/Wingless Signaling

Wnt/wingless signaling is one of the best-known pathways involved in the regulation of β-catenin protein levels. Knowing that β-catenin complexes with and that it's level is regulated by GSK-3, we examined the Wnt/wingless signaling associated with GSK-3 in the RIFLE–HA/PC12 cells. The Wnt/wingless gene family constitutes a large number of developmentally regulated genes involved in cell–cell signaling in a wide range of animal phyla. GSK-3α or β isoform, a major player in Wnt/wingless signaling, is a serine/threonine kinase known to directly regulate β-catenin levels by phosphorylating β-catenin on specific serine/threonine residues marking β-catenin for ubiquination. Cells expressing RIFLE consistently accumulated β-catenin while stimulating the phosphorylation of both GSK-3α (Ser21) and GSK-3β (Ser9)



C

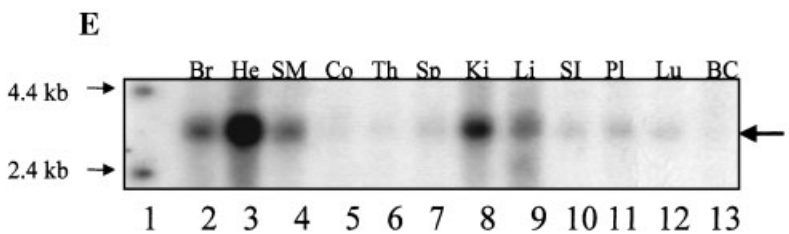
LRR motif in RIFLE

LRR	Amino acid sequence																						Residues
1	**L**	A	T	**I**	K	V	**L**	D	L	H	D	**N**	Q	**L**	T	A	**L**	P	D	D	L	G	Q	59-81
2	**L**	T	A	**L**	Q	V	**L**	N	V	E	R	**N**	Q	**L**	M	Q	**L**	P	R	S	I	G	N	82-104
3	**L**	T	Q	**L**	Q	T	**L**	N	V	K	D	**N**	K	**L**	K	E	**L**	P	D	T	V	G	E	105-127
4	**L**	R	S	**L**	R	T	**L**	N	I	S	G	**N**	E	**I**	Q	R	**L**	P	Q	M	L	A	H	128-150
	L	*X*	*X*	**L**	*X*	*X*	**L**	*X*	*X*	*X*	*X*	**N**	*X*	**L**	*X*	*X*	**L**	P	*X*	*X*	*X*	*X*	*X*	Consensus sequence

D

$\mathbf{Lx_2Lx_2Lx_4Nx_1L}$ RIFLE Consensus

$\mathbf{Lx_2Lx_1Lx_2Nx_1L}$ LRR Consensus



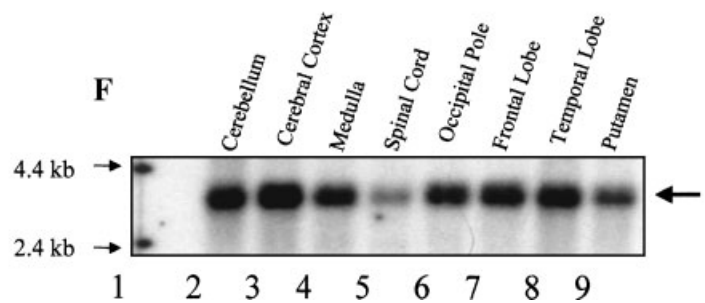


Fig. 1. (*Continued*)

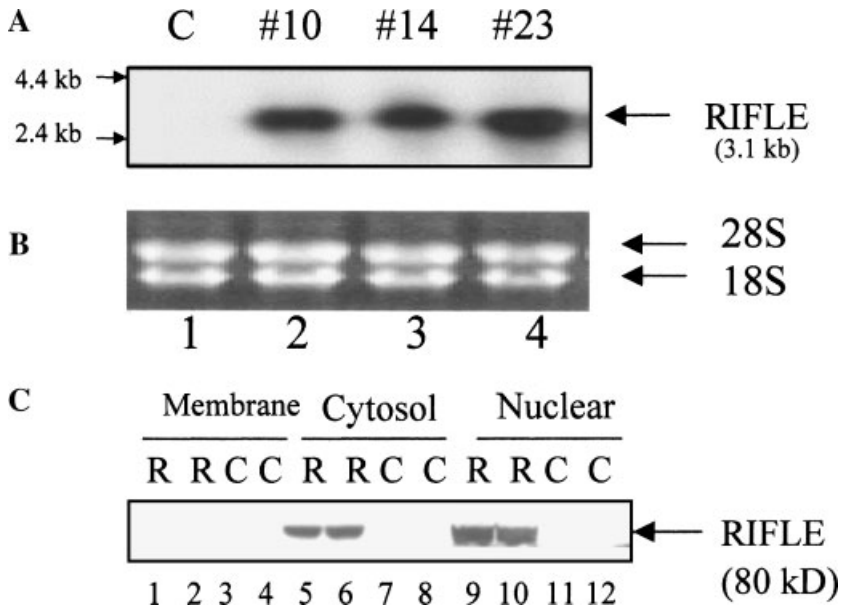


Fig. 2. **Panel A**: Expression of RIFLE in PC12 cells. RNA blot analysis of 25 μg of total RNA isolated from RIFLE transfected PC12 cell colony #10, #14, and #23, respectively. **Lane 1**: PC12 cell stably transfected with *pBI–EGFP* vector as a control. **Lanes 2–4** represent three independent clones #10, #14, and #23 of PC12 cells stably transfected with *pBI–RIFLE*. **Panel B** is an ethidium bromide stained RNA gel showing the equivalent loading of the RNA in the corresponding RNA blot. **Panel C**: Subcellular distribution of RIFLE in PC12 cells stably transfected with *pcDNA3.1–RIFLE–HA*. Shown is a Western blot probed with HA-tag antibody. Samples were analyzed in duplicate. Lanes 1 & 2, **5** & **6**, **9** & **10** (labeled with R) are PC12 cells expressing RIFLE. Lanes 3 & 4, **7** & **8**, **11** & **12** (labeled with C) are PC12 cells stably transfected with pcDNA3.1 vector as controls. RIFLE is predominantly expressed in the cytosol and nuclear fractions.

with predominant phosphorylation occurring on GSK-3α (Fig. 5A). This phosphorylation occurred without change in GSK-3α or β protein levels (Fig. 5B) or phosphorylation of several other kinases such as PKCβ (Fig. 5C), PKCα, PKCε, Erk1, or Erk2 (data not shown). Phosphorylation (Ser21/9) of GSK-3α and β were quantified by densitometry analysis in Figure 5D,E, respectively. RIFLE significantly stimulated an increase in GSK-3α/β phosphorylation (Ser21/9) of, most notably, GSK-3α.

Since increased Ser21/9 phosphorylation of GSK-3 is known to inhibit its kinase activity, this data suggested that RIFLE conceivably regulates β-catenin levels via inhibition of GSK-3 kinase activity, resulting in increased β-catenin. To determine if RIFLE inhibits GSK-3 kinase activity in the RIFLE–HA/PC12 cells, we next carried out experiments to directly measure the GSK-3 kinase activity. GSK-3 activity was measured in RIFLE and control PC12 cells using a specific GSK-3 CREB peptide substrate and our data, derived from five independent experiments, showed that GSK-3 kinase activity was significantly suppressed by RIFLE expression in the PC12 cells (Fig. 5F). This is in full agreement with the increase in Ser21/9-phosphorylation of GSK-3 by RIFLE and correlates with the observed β-catenin stability.

Elevation of β-Catenin/E-Cadherin Levels by RIFLE Is Accompanied by Increases in Cell Adhesion

Based on the fact that RIFLE up-regulates some adhesion-related molecules, we investigated RIFLE's effect on cell–cell and cell–matrix adhesions. We found that RIFLE expressing PC12 cells show increased cell–cell adhesion forming clusters in the presence of Ca^{++} (Fig. 6A) while control cells grew in a dissociated pattern and remained in a single cell pattern (Fig. 6B) in the cell–cell adhesion assay. Our data demonstrates that the expression of RIFLE significantly increases the Ca^{++}-mediated cell–cell adhesion as compared to control PC12 cells or to the RIFLE PC12 cells in the absence of Ca^{++} (plus EGTA) (Fig. 6C), suggesting that RIFLE mediates a Ca^{++} dependent cell–cell adhesion event. In order to directly address whether the observed cell–cell adhesion in RIFLE–HA/PC12 cells results from RIFLE

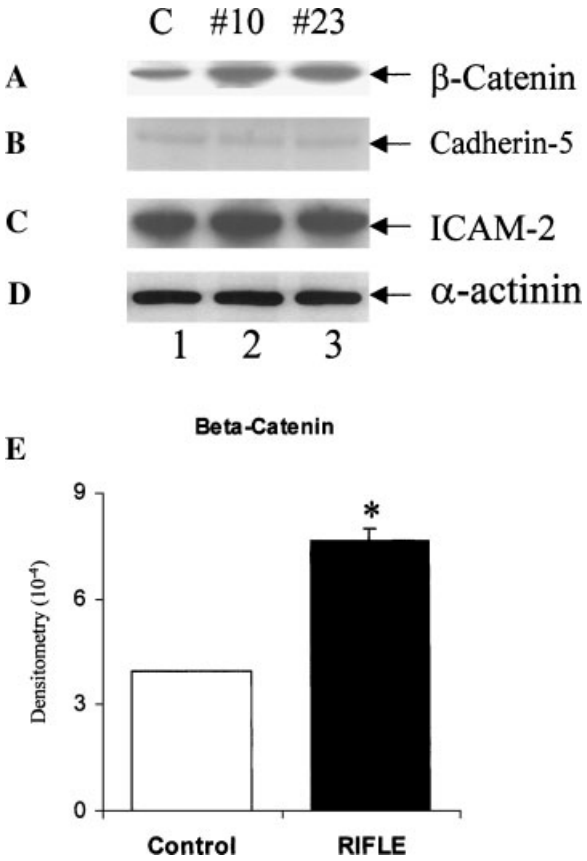


Fig. 3. Overexpression of RIFLE results in elevated protein levels of β-catenin in PC12 cells. **Panels A–D** are Western blots probed with antibodies against β-catenin, cadherin-5, ICAM-2, and α-actinin, respectively. α-Actinin blot was served as loading control. **Lane 1** is PC12/*pBI–EGFP* control; **lanes 2** & **3** are clone #10 and #23 of PC12/*pBI–RIFLE*. **Panel E** is a statistical analysis of β-catenin expression levels following overexpression of RIFLE. Densitometry of corresponding bands was measured; data from three independent cell lines of three experiments were pooled for statistical analysis ($P < 0.05$).

elevated E-cadherin levels, we carried out E-cadherin neutralization experiments using an anti-E-cadherin antibody. Indeed, E-cadherin antibody blocked the formation of cell–cell adhesion clusters in RIFLE–HA/PC12 cells in the presence of Ca^{++} (Fig. 6E) while antibody control IgG had no effect under the same conditions (Fig. 6D), suggesting that elevated cell–cell adhesion in RIFLE–HA/PC12 cells is E-cadherin mediated. Quantitative and statistical comparison of the antibody neutralization experiment is shown in Figure 6F. We also assessed whether RIFLE enhances cell–matrix adhesion in PC12 cells. Our data revealed that RIFLE enhanced the cell attachment to the ECM molecules collagen IV (Fig. 6G) and fibronectin (Fig. 6H).

Finally, we used a GSK-3β kinase deficient mutant to mimic RIFLE's effect on β-catenin/E-cadherin and cell adhesion. We made several kinase dead (KK) and vector stable PC12 cell lines for use in studying GSK-3β kinase deficient effects in PC12 cells. We found that the GSK-3 kinase dead mutants increased β-catenin protein (Fig. 7A) and E-cadherin protein (Fig. 7B) as compared to the vector control, mimicking RIFLE's effects in augmenting β-catenin stability and E-cadherin protein levels. Endogenous cdk5 protein serves as a loading control in Figure 7A,B. The quantitative elevation of β-catenin, GSK-3β, and E-cadherin are, after being normalized with cdk5 protein levels, shown in Figure 7C–E, respectively. Similarly, we also used lithium, a GSK-3 inhibitor. We determined the optimal dose of lithium for PC12 cells without affecting cell survival to be 25 mM (data not shown). Naive PC12 cells were treated with lithium at 25 mM for up to 24 h and the cells were then collected and prepared for β-catenin/E-cadherin expression analysis. β-Catenin and E-cadherin protein levels were increased 8 h after lithium treatment and remained at the same level up to 24 h (Fig. 7F,G). α-Actinin levels served as a loading control (Fig. 7H). Elevated protein levels of β-catenin and E-cadherin were quantified by densitometry analysis, normalized with α-actinin, and shown in Figure 7I,J, respectively.

GSK-3 kinase dead stable lines were subjected to both cell–cell and cell–matrix adhesion analysis. The stable PC12 cells expressing the GSK-3 kinase deficient mutant significantly enhanced the cell–cell adhesion in the presence of Ca^{++} as compared to their corresponding controls (without Ca^{++} and plus EGTA) (Fig. 8A). Interestingly, the GSK-3 kinase dead stable cell lines, unlike RIFLE expressing cells, failed to enhance cell–matrix adhesion on either collagen IV (Fig. 8B) or fibronectin (Fig. 8C). This data indicates that inhibition of GSK-3 kinase activity alone is sufficient to regulate β-catenin and E-cadherin protein levels and related cell–cell adhesion but not cell–matrix adhesion. Therefore, our data suggests that inhibition of GSK-3 kinase activity and associated elevation of β-catenin/E-cadherin protein levels by RIFLE contribute to RIFLE-mediated cell–cell adhesion as with Wnt/wingless signaling. RIFLE mediated cell–matrix adhesion may depend on a yet unexamined signaling pathway(s) more closely related to cell–matrix adhesion.

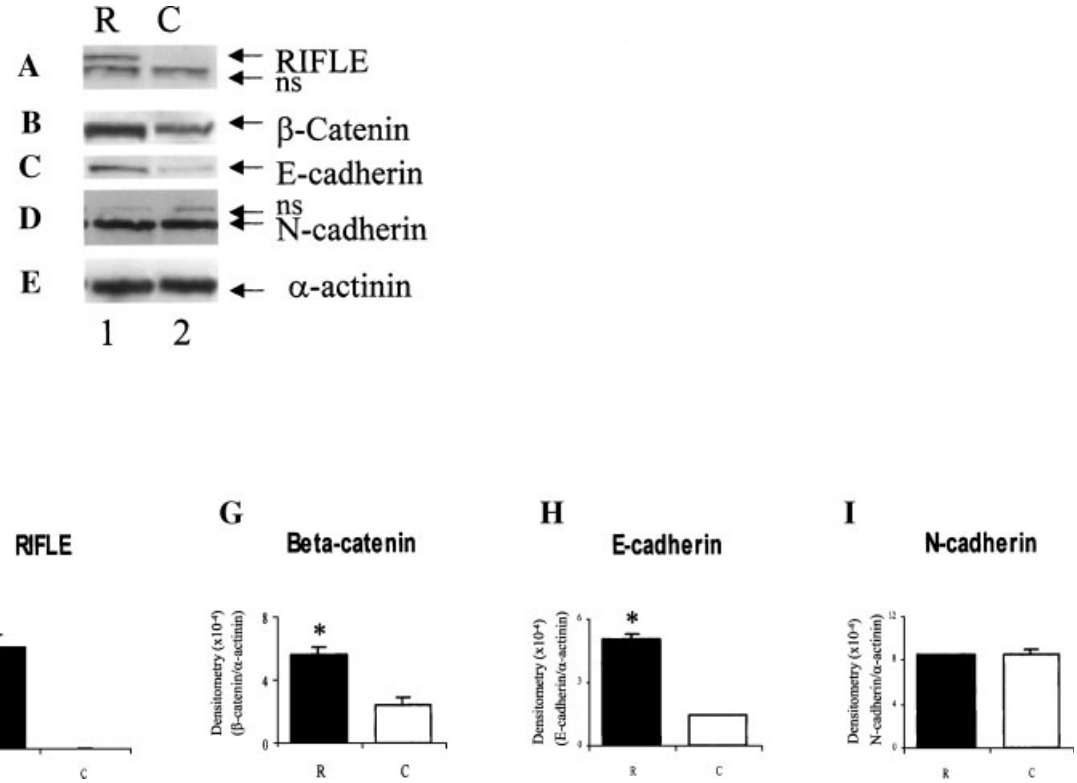


Fig. 4. RIFLE expression in PC12 cells enhances β-catenin and E-cadherin protein levels. **Panels A–E** are parallel-loaded blots subject to Western analysis with antibodies against HA (for RIFLE), β-catenin, E-cadherin, N-cadherin, and α-actinin, respectively, as indicated. **Lane 1** is RIFLE–HA PC12 cells and **lane 2** is vector/PC12 cells. E-cadherin expression but not N-cadherin is co-regulated with the β-catenin in response to RIFLE expression in the PC12 cells (n = 3). α-Actinin blot serves as a loading control. Non-specific binding is indicated by ns. **Panels F–I** are quantitative comparisons pooled from triplicate experiments for RIFLE (F), β-catenin (G), E-cadherin (H), and N-cadherin (I), respectively, where R and C represent RIFLE and control, respectively. All quantitative data were normalized based on α-actinin loading control. *Indicates the significant differences of R vs. C (*$P < 0.05$ in F–H).

DISCUSSION

In this article, we identify and characterize a novel ring zinc finger-leucine-rich repeat containing protein, designated as RIFLE, and primarily focus on its biological functions in the regulation of cell adhesion molecules such as β-catenin and E-cadherin as well as cell–cell and cell–matrix adhesion in PC12 cells. Expression of RIFLE in the PC12 cells results in accumulation of β-catenin, a key mediator of the Wnt/wingless signaling pathway, presumably by negatively regulating GSK-3, enhancing Ser21 and Ser9 phosphorylation of GSK-3α and β, respectively, and eliciting an inhibitory effect on GSK-3 kinase activity. RIFLE also co-regulates β-catenin and E-cadherin expression without significant change in N-cadherin or cadherin-5. Consistent with RIFLE's enhancement of these cell adhesion molecules, RIFLE/PC12 cells augment cell adhesion without causing significant change in cell proliferation or cell survival. Moreover, the effect on β-catenin stabilization, elevated E-cadherin expression, and cell–cell adhesion by RIFLE can be mimicked by lithium, a well-known GSK-3 inhibitor, or kinase deficient GSK-3 mutants (KK) in PC12 cells. Our data suggest that elevation of β-catenin protein with co-regulation of E-cadherin via inhibition of GSK-3 kinase activity is sufficient for the observed cell–cell adhesion but not for the cell–matrix adhesion mediated by RIFLE. Taken together, we conclude that RIFLE is a novel ring zinc finger-leucine-rich repeat containing protein that mediates components of Wnt/wingless signaling and cell–cell adhesion in PC12 cells. A proposed model for how RIFLE may regulate cell–cell or cell–matrix adhesion was summarized in Figure 9.

There is a similarity between Wnt-1 and RIFLE signaling in PC12 cells. First, both lead to suppression of GSK-3 kinase activity

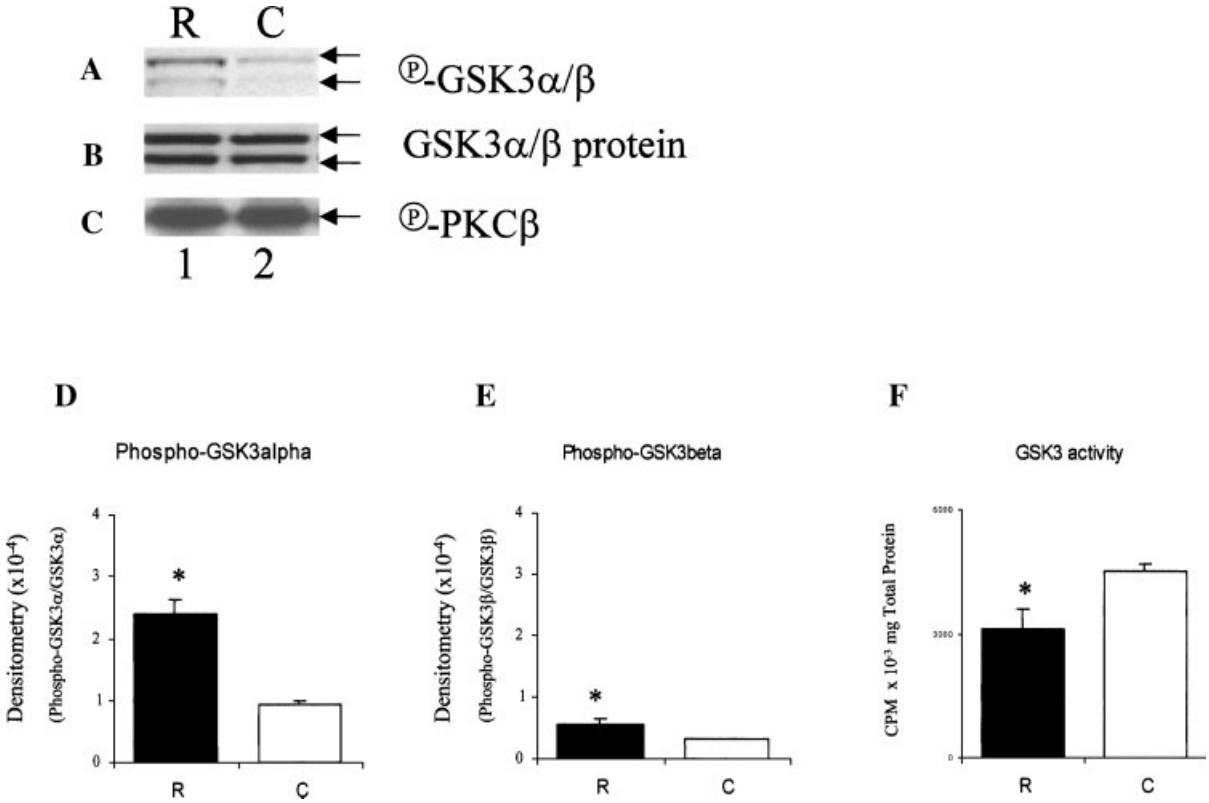


Fig. 5. Overexpression of RIFLE in PC12 cells increases the phosphorylation at Ser21/9 of GSK-3α/β and results in lower GSK-3 kinase activity. **Panels A–C** are parallel loaded gels subjected to Western blot analysis with antibodies against phospho-GSK-3α/β, GSK-3α/β protein, and phospho-PKCβ, respectively as indicated. **Lane 1** is RIFLE–HA/PC12 cells and **lane 2** is vector/PC12 cells. **Panels D** and **E** are quantitative comparisons pooled from triplicate experiments for phospho-GSK-3α (D) and phospho-GSK-3β (E), respectively, where R and C represent RIFLE and control, respectively. The quantitative data for phospho-GSK-3α/β were normalized with GSK-3α/β protein levels. *Indicates the significant differences of R vs. C ($*P < 0.01$ in panel D; $*P < 0.05$ in **panel E**). Panel F shows significant inhibition of GSK-3 kinase activity in RIFLE–HA/PC12 cells (black bar) compared to vector transfected PC12 control cells (white bar). Data represents the average from five independent experiments ($*P < 0.05$).

increasing β-catenin protein levels, a key mediator of the Wnt/wingless signaling pathway, and both elevate E-cadherin protein [Bradley et al., 1993; Hinck et al., 1994; Hollmann et al., 2001]. The selective regulation of RIFLE on GSK-3 kinase activity and cell adhesion molecules is evident and robust. We examined over 10 other closely related cell adhesion-related molecules such as P-cadherin, N-cadherin, and cadherin-5 and a variety of different kinases such as Erk1, Erk2, PKCα, PKCβ, and PKCε and found that they are not regulated by RIFLE. Interestingly, RIFLE appears to have some selectivity for GSK-3α over the β isoform, evidenced by the enhanced phosphorylation of the GSK-3α isoform. There is considerable evidence that Wnt/wingless signaling is a conserved regulatory pathway for GSK-3. For example, the mammalian homolog of Sgg^{Zw3}, GSK-3, has been shown to be inhibited by *Drosophila* Wg protein in fibroblasts [Cook et al., 1996] and embryos [Ruel et al., 1999]. Further, expression of Wnt-1 in the PC12 cells leads to increases in β-catenin, E-cadherin, and consequent cell adhesion [Bradley et al., 1993; Hollmann et al., 2001]. Although the precise mechanism underlying the co-regulation of β-catenin and E-cadherin is not clear, a variety of studies suggest that there is a cross talk mechanism by which expression of these two molecules are co-regulated. Indeed, overexpression of the E-cadherin, for example, results in the up-regulation of three major catenins including β-catenin in the E-cadherin-negative Dunning clones [Luo et al., 1999]. Interestingly, suppression of basal extracellular signal-regulated kinase (Erk) activity in PC12 cells results in the up-regulation of both cadherin and β-catenin, enhancing cell adhesion [Lu et al., 1998]. The fact that either lithium or the kinase

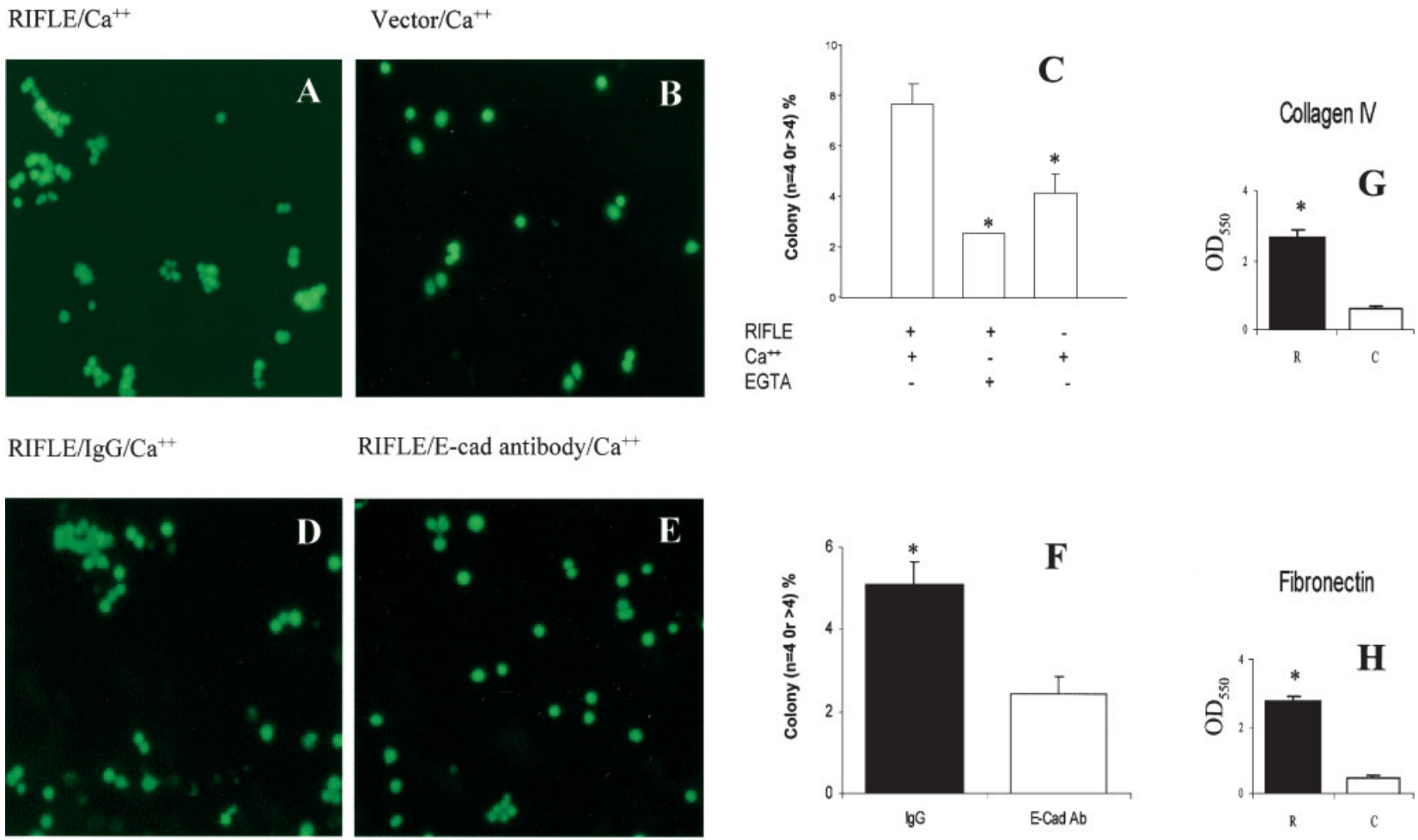


Fig. 6. Overexpression of RIFLE in PC12 cells increases both cell–cell and cell–matrix adhesion. **Panel A** shows representative cell–cell clusters formed in RIFLE–HA/PC12 cells in the presence of Ca^{++}. **Panel B** shows the single or two cell patterns seen in vector/PC12 cells in the presence of Ca^{++}. **Panel C**: The quantitative comparison of cell–cell adhesion in PC12 cells transfected with or without RIFLE in the presence or absence (plus EGTA) of Ca^{++}. Shown are the representative results of three repeated experiments ($^*P < 0.01$). **Panels D** and **E** show that E-cadherin antibody neutralized the cell–cell adhesion (E) in RIFLE–HA/PC12 cells while antibody control IgG (D) did not. **Panel F** shows the quantitative comparisons of cell–cell adhesion represented in panels D and E ($^*P < 0.01$). **Panels G** and **H** show the quantitative analysis of cell–matrix adhesion (collagen IV, panel G; fibronectin, panel H) of PC12 cells expressing RIFLE. C and R represent control and RIFLE, respectively ($^*P < 0.01$).

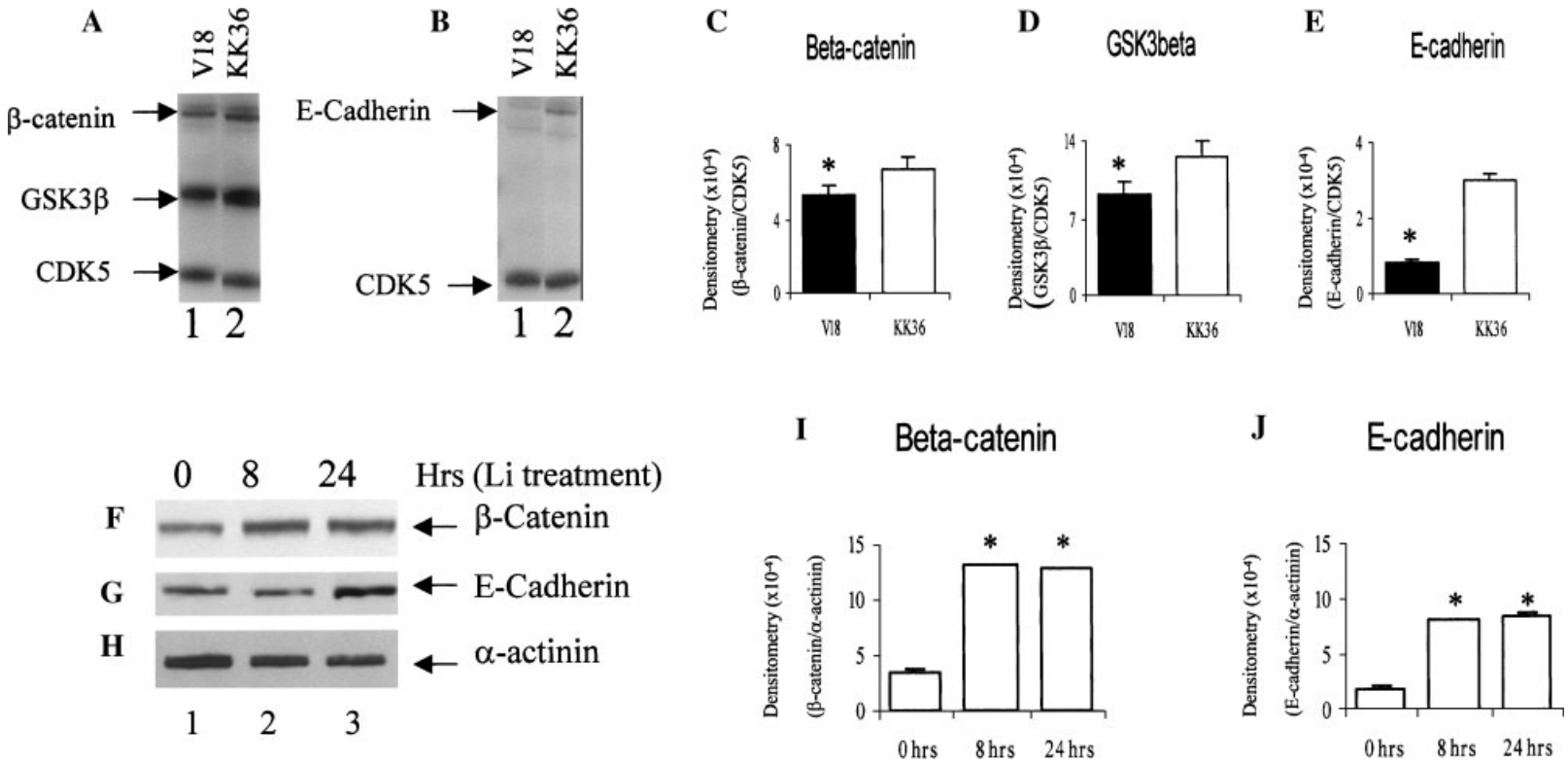


Fig. 7. Overexpression of kinase-deficient GSK-3β mutant (KK) construct results in accumulation of β-catenin and E-cadherin in naive PC12 cells. **Panels A** and **B**: PC12 cells stably transfected with vector construct (**lane 1**) and the kinase-deficient GSK-3β mutant (KK) construct (**lane 2**) were subjected to Western blot analysis with antibodies against β-catenin/GSK-3β/CDK5 (A) and E-cadherin/CDK5 (B), respectively. CDK5 protein serves as a loading control in both panels A and B. **Lanes 1** and **2** of panel A and B are PC12 cells stably transfected with vector (clone #18) and kinase-deficient GSK-3β mutant (KK, clone # 36), respectively. Protein levels of β-catenin and GSK-3β in panel A and E-cadherin in panel B were quantified by densitometry analysis normalized with CDK5 protein levels as shown in **panels C–E**. **Panels F–H**: Parallel loaded gels with samples prepared from naive PC12 cells treated with lithium (25 mM) at times indicated, were subjected to Western blot analysis with antibodies against β-catenin, E-cadherin, and α-actinin as indicated. Panel H (α-actinin) serves as loading control. Lanes 1–**3** are cells harvested 0, 8, and 24 h following lithium treatment. The kinetic changes on protein levels of β-catenin and E-cadherin were quantified by densitometry analysis ($n = 3$) normalized with α-actinin protein levels as shown in **panels I** and **J**, respectively. *Indicates significant difference of 0 h vs. 8 and 24 h in panels I and J ($P < 0.05$).

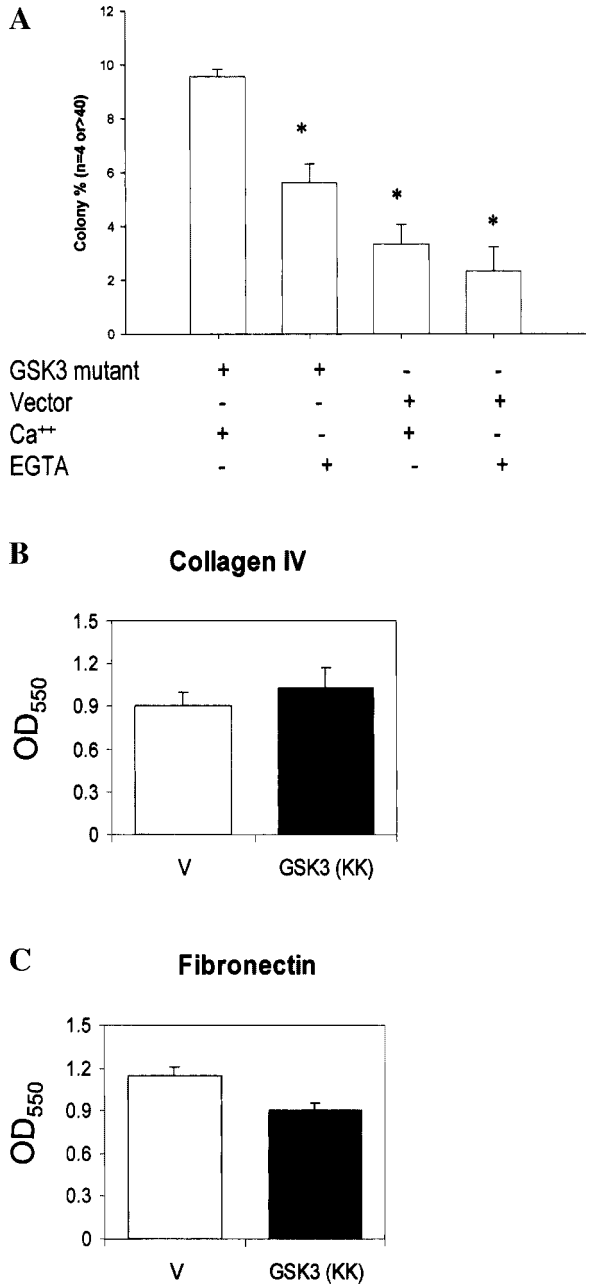


Fig. 8. Overexpression of kinase-deficient GSK-3β (KK) in naïve PC12 cells mimics the RIFLE mediated effects in cell–cell adhesion but not cell–matrix adhesion. **Panel A**: Cell–cell adhesion of the GSK-3β kinase dead (KK)/PC12 cells in the presence or absence (plus EGTA) of Ca^{++}. The stable PC12 cells expressing GSK-3β (KK) significantly enhanced the cell–cell adhesion as compared to the multiple controls indicated. **Panels B** (collagen IV) and **C** (fibronectin) show no effect of expressed GSK-3β dead kinase on PC12 cell–matrix adhesion ($n = 3$, $^*P < 0.05$).

deficient GSK-3 mutant can independently mimic the effect of RIFLE or Wnt-1 on GSK-3β-catenin/E-cadherin pathway suggests that inhibition of GSK-3 alone is sufficient in leading to the subsequent elevation of β-catenin and E-cadherin.

Secondly, both Wnt/wingless and RIFLE signals enhance cell adhesion. Overexpression of Wnt-1 results in an apparent increase in cell–cell adhesion via increase in β-catenin/E-cadherin [Bradley et al., 1993; Bournat et al., 2000]. Overexpression of RIFLE substantially increases Ca^{++} dependent cell adhesion, presumably via a similar mechanism through β-catenin/E-cadherin. The classic cadherins with a single transmembrane domain are known to be involved in Ca^{2+}-dependent cell–cell adhesion. Interestingly, RIFLE enhances not only cell–cell adhesion but also cell–ECM adhesion to collegen IV and fibronectin. It is unclear if Wnt-1 can enhance cell–matrix adhesion, but Wnt-5a protein was shown to participate in regulation of cell to collagen adhesion via discoidin domain receptor 1 (DDR1) [Jonsson and Andersson, 2001]. It is conceivable that RIFLE regulates cell–cell and cell–matrix through different signaling pathways. One clearly being the GSK-3/β-catenin/E-cadherin signaling pathway associated with cell–cell adhesion, a notion that is supported by increases in Ca^{++} dependent cell–cell adhesion following inhibition of GSK-3 kinase activity by RIFLE or by lithium or kinase deficient GSK-3 mutants. The other probably by a GSK-3 unrelated signaling mechanism(s) since inhibition of GSK-3 alone at least by the GSK-3 (KK) mutant is unable to enhance the cell–matrix adhesion like that observed in the RFILE/PC12 cells. We, however, cannot exclude the possibility that the regulation of β-catenin/E-cadherin by RIFLE may facilitate the cell–matrix adhesion process. Indeed, it has been reported that the E-cadherin/β-catenin complex is able to regulate both cell–cell and cell–matrix adhesion with Ezrin [Hiscox and Jiang, 1999].

Despite the similarity between RIFLE and Wnt/wingless signaling, signaling mediated by RIFLE is clearly different. For example, RIFLE does not significantly influence PC12 cell differentiation and neurite outgrowth in response to NGF stimulation whereas expression of the Wnt-1 oncogene in PC12 cells does induce morphological and biochemical changes, including lack of differentiation in response to growth factors [Bournat et al., 2000]. Although the precise mechanism by which RIFLE inhibits GSK-3 activity is unclear, RIFLE appears not to impact on PKCα, β, and ε activity while Wnt/

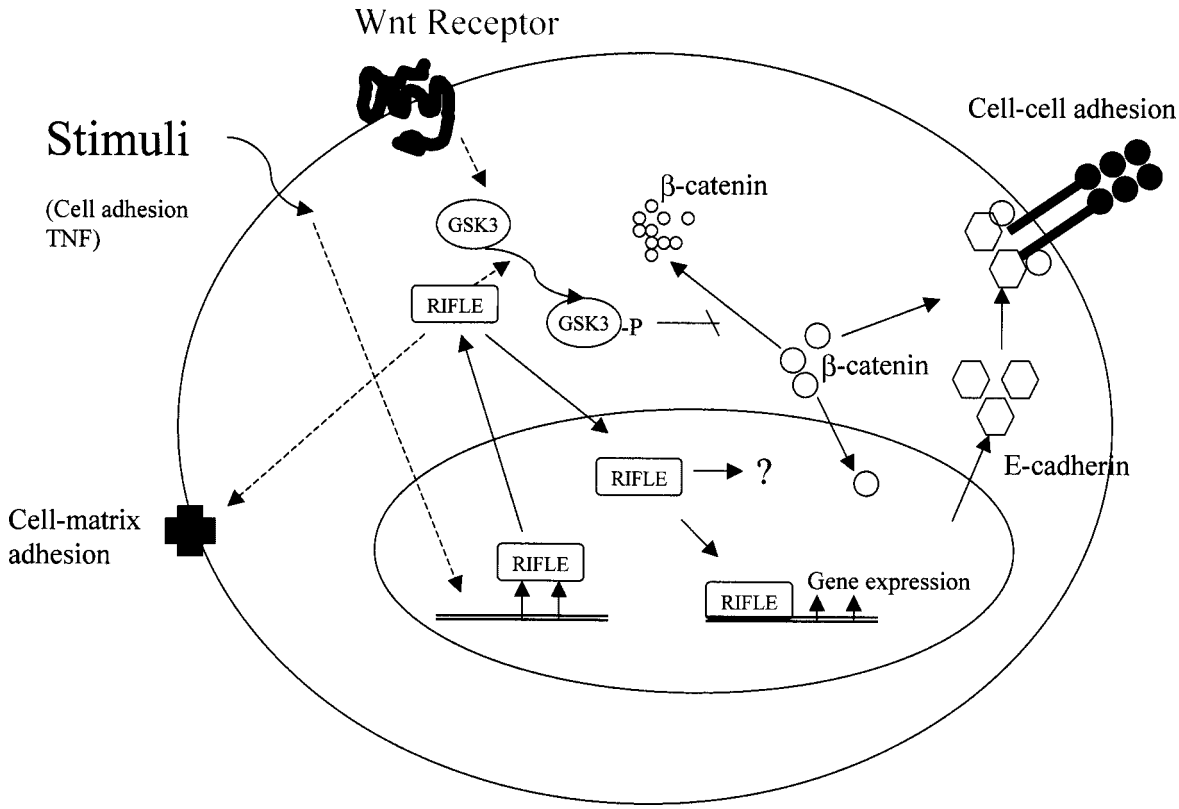


Fig. 9. We postulated that expression of RIFLE plays an important role in cell–cell or cell–matrix adhesion. In this model, RIFLE could be induced by extracellular stimuli, e.g., by cell adhesion and cytokines, and exerts its functions in cytosol and nuclei. In cytosol, RIFLE may regulate the phosphorylation of GSK-3, thus GSK-3 activity, resulting in stabilization of β-catenin. In nuclei, RIFLE, which contains DNA binding domain, could serve as a transcriptional factor regulating gene expression such as E-cadherin. Increase in β-catenin and E-cadherin facilitate the cell–cell adhesion. Expression of RIFLE could facilitate cell–matrix adhesion via a yet unknown mechanism.

wingless signaling abrogates GSK-3 kinase activity via an intracellular pathway involving PKC [Cook et al., 1996].

In conclusion, RFILE represents as a novel protein that regulates cell adhesion molecules, such as β-catenin and E-cadherin, and cell adhesion in PC12 cells. GSK-3 is a critical component in the RIFLE signaling cascade, particularly in the RIFLE-mediated cell–cell adhesion. Our study suggests a new mechanism/pathway by which a novel protein, RIFLE, signals involving crosstalk between components of Wnt/wingless and cell adhesion.

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