THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 1, Issue of January 4, pp. 303–309, 2002
Printed in U.S.A.

Reelin Is a Serine Protease of the Extracellular Matrix*

Received for publication, July 24, 2001, and in revised form, October 18, 2001
Published, JBC Papers in Press, October 31, 2001, DOI 10.1074/jbc.M106996200

Carlo C. Quattrocchi,[a,b,c] Francesca Wannenes,[d,e] Antonio M. Persico,[a] Silvia Anna Ciafré,[d] Gabriella D'Arcangelo,[f,g,h,i] Maria G. Farace,[f] and Flavio Keller[a,j]

From the [a]Laboratory of Neuroscience, Department of Physiology and Neuroscience, Università "Campus Bio-Medico," Via Longoni 83, 00155 Roma, Italy, the [b]Program in Neuroscience, Faculty of Medicine, University of Brescia, Via Valsabbina 19, 25123 Brescia, Italy, the [d]Department of Experimental Medicine and Biochemical Sciences, Università di Tor Vergata, Via di Tor Vergata 135, 00133 Roma, Italy, the [e]Department of Internal Medicine, Università di Tor Vergata, Via di Tor Vergata 135, 00133 Roma, Italy, and [f]The Cain Foundation Laboratories, [g]Department of Pediatrics, [h]Program in Developmental Biology and [i]Division of Neuroscience, Baylor College of Medicine, Houston, Texas 77030

Reelin is an extracellular matrix protein that plays a pivotal role in development of the central nervous system. Reelin is also expressed in the adult brain, notably in the cerebral cortex, where it might play a role in synaptic plasticity. The mechanism of action of reelin at the molecular level has been the subject of several hypotheses. Here we show that reelin is a serine protease and that proteolytic activity is relevant to its function, since (i) Reelin expression in HEK 293T cells impairs their ability to adhere to fibronectin-coated surfaces, and adhesion to fibronectin is restored by micromolar concentrations of diisopropyl phosphorofluoridate, a serine hydrolase inhibitor; (ii) purified Reelin binds FP-Peg-biotin, a trap probe which irreversibly binds to serine residues located in active catalytic sites of serine hydrolases; (iii) purified Reelin rapidly degrades fibronectin and laminin, while collagen IV is degraded at a much slower rate; fibronectin degradation is inhibited by inhibitors of serine proteases, and by monoclonal antibody CR-50, an antibody known to block the function of Reelin both *in vitro* and *in vivo*. The proteolytic activity of Reelin on adhesion molecules of the extracellular matrix and/or receptors on neurons may explain how Reelin regulates neuronal migration and synaptic plasticity.

Reelin (1, 2) is an extracellular matrix protein that plays a pivotal role in neuronal migration during development of laminar structures of the mammalian brain including the cerebral cortex, hippocampus, cerebellum, and several brainstem nuclei, as shown by spontaneous Reelin null mutations (*i.e.* the *reeler* mouse) (3, 4). In the developing cerebral cortex, Reelin is secreted by Cajal-Retzius cells, located in the marginal zone. Reelin must be secreted into the extracellular matrix to exert its biological effect (5).

In the *reeler* mouse, migrating neurons fail to pass through earlier-generated neurons, possibly because they are unable to penetrate the subplate, or because they maintain extensive contacts with the radial glial fibers (6). Several hypotheses have been suggested regarding the function of Reelin: (i) Reelin may act as an attractant molecule for migrating neurons; (ii) it may act as a repulsive molecule; or (iii) Reelin may interrupt the association between migrating neurons and radial glia (7, 8), thus allowing migrating neurons to switch from a "gliophilic" to a "neurophilic" state (9). Furthermore, Reelin has been recently shown to be expressed in several adult neuronal cells, including glutamatergic cerebellar granule neurons and specific GABAergic interneurons of the cerebral cortex and hippocampus (10), and in the adult mammalian blood, liver, pituitary pars intermedia, and adrenal chromaffin cells (11, 12). The cellular function of Reelin in the adult organism is unknown. Evidence is accumulating for involvement of Reelin in human diseases such as autosomal recessive lissencephaly (13), schizophrenia (14), and autistic disorder (15).

The mouse Reelin sequence (1) encompasses 3461 amino acids and possesses a signal peptide followed by a domain with 28% sequence identity with F-spondin (as assessed by Ψ-Blast software), a protein secreted by floor plate cells and promoting cell adhesion and neurite growth (16). This region is followed by a unique region with no sequence homology, and then by eight internal repeats of 350–390 amino acids, each repeat containing two related subdomains flanking a cystein-rich sequence similar to the epidermal growth factor-like motif. The carboxyl terminus region contains many positively charged amino acids required for secretion (5). Human Reelin (2) is 94.8% identical to the mouse protein at the amino acid level, indicating strong functional conservation. Recent findings suggest that the Reelin signal transduction involves binding to the very low density lipoprotein receptor and to apoE receptor 2 followed by intracellular activation of the adapter protein disabled-1 (17–19). Other possible Reelin signal transduction pathways may involve interaction with the $\alpha_3\beta_1$ integrin receptor (8, 9) and with cadherin-related neuronal receptors (20).

We have analyzed the primary amino acid sequence of human Reelin, and found several hints that Reelin might be a serine protease, since: (i) Reelin contains the sequence GKSDG (amino acids 1280–1284 of human Reelin) (2), corresponding to the serine hydrolase consensus sequence G*X*S*X*G; this sequence is 100% conserved among mouse, chicken, and human Reelin; (ii) Reelin shows significant structural similarities with serine hydrolases, such as the extracellular serine protease precursor (EC 3.4.21) of *Serratia marcescens*, and the probable ubiquitin carboxyl-terminal hydrolases FAM and FAF-Y (EC 3.1.2.15); (iii) Reelin contains eight epidermal growth factor-like repeats; epidermal growth factor-like repeats are observed in serine proteases, for example, coagulation factors VII, IX, and X, and protein C, Z (21), calcium-dependent serine protein-

* This work was supported by Consiglio Nazionale delle Ricerche Programma "Biomolecole per la salute umana" Grant 99.00555.PF33. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[c] Present address: Dept. of Pediatrics-Neurology, Baylor College of Medicine, 1102 Bates St., MC 3–6365, Houston, TX 77030.

[j] To whom correspondence should be addressed: Laboratory of Neuroscience, Università "Campus Bio-Medico," Via Longoni 83, 00155 Roma, Italy. Tel.: 39-06-2254-1335; Fax: 39-06-22-54-14-56; E-mail: f.keller@unicampus.it.

ase (22) which degrades extracellular matrix proteins, and complement C1s and C1r components (23); (iv) several serine hydrolases, such as lipoprotein lipase and the urokinase-type plasminogen activator, bind very low density lipoprotein receptor and apoE receptor 2 (24, 25). In this study we present converging evidence that purified Reelin acts as a serine protease, and that this enzymatic activity may be relevant for its cellular function.

EXPERIMENTAL PROCEDURES

Chemicals—Cell culture media, antibiotics, and media supplements were purchased from Invitrogen (Gaithersburg, MD). All other chemicals were from Sigma, unless otherwise specified.

Amino acid Sequence Analysis of Reelin—All sequence analyses are based on human Reelin (accession number: NP005036) (2). Homologies were evaluated by using Ψ-Blast 2 sequences software (www.ncbi.nih.gov/blast). Also, PropSearch software (EMBL, Heidelberg) was used to find homologies with other serine hydrolases. PattinProt software (PBIL, NPSA, Lyon) was used to identify putative consensus sequences for serine proteases.

Cell Culture, Transfection, and Expression of Recombinant Reelin—Human embryonic kidney (HEK) 293T cells (ATCC, Q401) were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin/streptomycin (100 international units/ml and 100 μg/ml, respectively), and 10 mM L-glutamine. All cells were cultured at 37 °C, 5% CO_2 and 97% relative humidity. Passaging was routinely performed with trypsin-EDTA. Cells were stably transfected with 2 μg of plasmid DNA pCrl (5), which contains the entire mouse Reelin open reading frame (accession number NP_035391), or with 2 μg of pCDNA3 empty vector in 10 μl of LipofectAMINE in 4 ml of Opti-MEM serum-free medium, according to the manufacturer's instructions. Cells were transfected in 60-mm plates at a density of 5×10^5/dish and transferred to selection medium (0.6 mg/ml G418) 48 h after transfection. For Reelin purification, a stable cell line (CER) was generated by stable transfection of 293-EBNA cells (Invitrogen) with pCER followed by selection in medium containing 0.25 mg/ml G418 and 0.4 mg/ml hygromycin B. The pCER episomal plasmid contains a *reelin* insert identical to that of pCrl, cloned into the pCEP4 vector (Invitrogen).

Cultures were harvested for RNA preparation (26). Total RNA (3 μg) was incubated in reaction buffer containing 5 μM random hexamer (Amersham Bioscience, Inc.), Moloney murine leukemia virus reverse transcriptase, reaction buffer, and RNase inhibitor, according to the manufacturer's specifications (Invitrogen). Reverse transcription was performed for 1 h at 37 °C, and stopped by incubating the samples for 10 min at 95 °C. Expression of Reelin was checked by PCR amplification with specific primers: forward 5′-GGAAAGTCAGATGGAGAC-3′, reverse 5′-CATCTAAGCCAAACG-3′ corresponding to nucleotides 4123–4498 of mouse Reelin mRNA (Ref. (1), accession number U24703). PCR amplification was carried out in a total volume of 50 μl, with 10 μl of reverse transcription reaction and 1.25 units of *Taq* polymerase (M-Medical Genenco-Life Science). Amplification was performed for 35 cycles (1 min denaturing at 94 °C, 1 min annealing at 58 °C, and 1 min extension at 72 °C) in a thermal cycler (Oracle BioSystems, Delphi 1500). The 376-bp amplified product was analyzed by 2% agarose gel electrophoresis and visualized under UV illumination after staining with ethidium bromide. Reelin secretion into supernatants was analyzed by plating 1×10^6 cells onto 90-mm dishes. After 24 h conditioned media were removed, cells were washed twice with phosphate-buffered saline (PBS), and serum-free Dulbecco's modified Eagle's medium was added. Forty-eight hours later, the supernatants were collected, cleared by a brief centrifugation (10 min, 1000 rpm) at 4 °C, concentrated by Microcon YM-100 (Millipore Corp. Bedford, MA) and stored at −80 °C for analysis by SDS-PAGE and immunoblotting. Reelin secretion into the cell culture medium was assessed by Western blots using mAb 142, an antibody that recognizes the NH_2-terminal sequence of Reelin (27).

Cell Adhesion Assay—Cell adhesion assay was performed according to published protocols (28). Ninety-six-well plates were coated overnight with fibronectin (2.5 μg/ml) in carbonate/bicarbonate buffer, pH 9.7. Cells were harvested, washed three times with serum-free Dulbecco's modified Eagle's medium, and then resuspended in sterile attachment solution (calcium- and magnesium-free Hanks' balanced salt solution, 20 mM HEPES, 1 mg/ml heat-inactivated bovine serum albumin, 1 mM $CaCl_2$, 1 mM $MgCl_2$. Mock- and pCrl-transfected 293T cells (1×10^4 in 200 μl/well) were allowed to attach for 2 h at 37 °C in a humidified 5% CO_2 incubator. Unattached cells were removed with Hanks' balanced salt solution. Attached cells were fixed with 4% formaldehyde in PBS, pH 7.4, and counted manually using an inverted microscope by two different observers. The counting area was defined by a grid (12 mm^2 area) placed under the wells. Attached cells were defined as cells that had spread and grown at least 1 process. All data are expressed as mean ± S.E. Differences between groups were tested by one-way ANOVA followed by the LSD post-hoc test, using the SPSS statistics package (SPSS, Chicago, IL, version 9.0).

Gel Electrophoresis and Immunoblotting—Samples were mixed with 2 × sample buffer and heated at 100 °C for 2 min. Six percent resolving gel, pH 8.8, and 5% stacking gel, pH 6.8, were prepared and run in a MiniProtean II assembly (Bio-Rad, Hercules, CA) according to standard protocols (29). Proteins were transferred to nitrocellulose filter (Schleicher & Schuell, GmbH, Dassel, Germany) by a semi-dry blotting apparatus (Hoefer Scientific, Amersham Bioscience, Inc., San Francisco, CA) for 90 min at constant current of 70 mA. After blotting, the nitrocellulose filter was blocked for 30 min in tris-buffered saline with 0.1% Tween 20 (TBST), containing 1% bovine serum albumin. Reelin was revealed with 1:2000 mAb 142, overnight, followed by 1:5000 alkaline phosphatase-labeled anti-mouse IgG (Promega Italia, Milan, Italy). Filters were developed with nitro blue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate in Tris-HCl, pH 9.5, containing 5 mM $MgCl_2$. The reaction was stopped with PBS containing 2 mM EDTA. Alternatively, Reelin bands were revealed using a chemiluminescence method**:** filters were incubated with biotinylated anti-mouse secondary antibody (1: 25.000 in TBST) for 1 h. Then, after three washes, 1:500 peroxidase-conjugated streptavidin (CHEMICON Int., Temecula, CA) was added for 15 min. Finally filters was washed, incubated in a freshly made solution containing 200 mM Tris-HCl, pH 8.5, 250 mM 3-aminophthalhydrazide, 40 mM *p*-coumaric acid, and 0.0005% H_2O_2, dried, and exposed to Kodak X-OMAT film. Filters or films were scanned with a SCANJET ADF digital scanner using Twain-32 software (Hewlett-Packard). The specificity of staining was checked by preincubating mAb 142 with protein SP, the Reelin fragment recognized by mAb 142 (27).

Labeling of Reelin with FP-Peg-biotin—Fluorophosphate biotin (FP-Peg-biotin) (a gift of Dr. Benjamin Cravatt) (30), stored as a 100 mM stock solution in Me_2SO at −20 °C, was added directly to protein samples to a final concentration of 2–4 μM. The reaction mixture was incubated at room temperature for 30 min, and stopped by adding an equal volume of 2 × reducing sample buffer. As a control for the specificity of FP-Peg-biotin labeling, replica samples were incubated for 1 h with 11.4 μM diisopropylphosphofluoridate (DFP),[1] a potent and specific serine-hydrolase inhibitor, before incubation with FP-Peg-biotin. Samples were separated by SDS-PAGE and transferred by electroblotting to nitrocellulose membranes; the membranes were blocked in TBST with 3% bovine serum albumin for 1 h at 25 °C or overnight at 4 °C, and then incubated for 15 min with an avidin-horseradish peroxidase conjugate (Pierce) diluted 1:300 in blocking solution. The labeled bands were revealed by chemiluminescence (see above).

Purification of Reelin—For gel filtration chromatography purification**,** 50 μl of supernatant from Reelin secreting CER cells were concentrated first by osmotic dyalisis with AQUACIDE I (Calbiochem, La Jolla, CA). A 5-ml concentrated sample was loaded on a FPLC system ÄKTAprime and passed through a HiLoad Superdex 200 26/60 column (Amersham Bioscience, Inc., Uppsala, Sweden). The run was performed at a constant flow rate of 3 ml/min and maximal pressure limit of 0.5 Pa. The eluate was collected in 1.5-ml fractions. After dot immunoblot screening and Western blot, positive fractions for Reelin were pooled and concentrated again as described above.

For purification by SDS-PAGE, Reelin-containing supernatant from transfected 293T cells was concentrated as above and separated on a 5% gel. The gel was run for 3 h 30 min at 125 V to achieve a good separation in the >250 kDa range. Thereafter a thin vertical slice of gel was cut and stained with silver to reveal the 400-kDa Reelin band. A 5-mm wide horizontal slice was cut from the remaining gel, using the stained slice as a reference, and 400-kDa Reelin was electroeluted at 60 V into 25 mM Tris, 250 mM glycine, 0,1% SDS buffer. Electroelution was carried out overnight at 4 °C. Purified Reelin was, then, transferred to PBS, pH 7.2, by overnight dialysis in a Slide-A-Lyzer cassette (Pierce). Finally the protein was concentrated. The final protein concentration was estimated to be 0.2 μg/ml (Bradford Reagent, Sigma). Purified Reelin was re-electrophoresed on a SDS gel to check the purity of the protein.

[1] The abbreviations used are*:* DFP, diisopropylphosphorofluoridate; HEK, human embryonic kidney; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; tPA, tissue plasminogen activator.

Aminoacid		Homology	Serine Protease Family
Ser	aa1280-1284 G K S D G	100%	Serine hydrolase
	Consensus s. *G X S X G*		
	aa441-452 E C G T I E S G L S M V	62%	Trypsin
	aa594-605 S T N H G R S W S L L H	63%	
	aa1097-1108 G C G V I S S G S S L Y	66%	
	Consensus s. *D G X X G D S G G S L L* *N S E S A I I* *S T P V V* *T A H M M* *A P V F F* *G I Y Y* *C M W S* *V H T* *Q A* *H N* *Q* *H*		
His	aa159-164 A T A T H R	63%	Trypsin
	Consensus s. *L S A S H C* *I T T* *V A* *M G*		
	aa1419-1429 H G D C I S G V C F C	68%	Subtilisin
	aa2060-2070 H S S S H V S S L C S	65%	
	aa3238-3248 H G Y C T T G A I C I	68%	
	Consensus s. *H G S X V S G X L S S* *T I T S I T A* *M C A V A G* *G M G* *C A C* *L* *V*		
Asp	aa1938-1949 I W I V D D F I I D G N	80%	Subtilisin
	aa1775-1786 R G I C D A G R C V C D	73%	
	aa2398-2408 E Y S V D L G L S W - H	73%	
	Consensus s. *S X L L D D G L X(2-3) D* *T I I S I N* *A V V T V H* *I M M A M* *V F F* *C*		

FIG. 1. **Consensus sequence analysis of Reelin around hypothetically catalytic amino acids (serine, histidine, and aspartic acid) of serine proteases.** Identical or homologous residues are *shaded*.

Degradation of Extracellular Matrix Proteins—Reelin aliquots (10 ng) were incubated with 1 μg of fibronectin from human plasma (Sigma), or with laminin or collagen type IV from basement membrane of Engelbreth-Holm-Swarm mouse sarcoma (Sigma) for 0, 10, 30, or 120 min at 37 °C, in PBS, pH 7.9. The reaction was stopped by adding sample buffer and heating the samples at 100 °C for 2 min. Samples were separated in a 8% SDS gel. After electrophoresis, the gel was fixed, and silver-stained.

RESULTS

Reelin Contains Regions of Homology with Serine Proteases—Human Reelin contains the sequence GKSDG (amino acids 1280–1284), homologous to the consensus sequence G*X*-S*X*G of serine proteases (Fig. 1). Furthermore, searches of the Reelin sequence for consensus patterns (PROSITE www.ich/ucl.ac.uk/cmgs/serpro.htm) around hypothetical amino acids of the catalytic triad (Ser, His, and Asp) using PattinProt (PBIL, NPSA, Lyon), yielded several sequences sharing >60% homology with serine proteases (Fig. 1).

Reelin Inhibits Cell Attachment in Vitro—To obtain recombinant Reelin protein, HEK 293T cells were transfected with pCrl plasmid, and Reelin mRNA expression was assessed by RT-PCR, using primers complementary to exon 27 sequences. RT-PCR of pCrl-transfected cells revealed the expected 376-bp band (Fig. 2*A*, *lane 3*), while the band was absent in mock-transfected cells (*lane 2*). Reelin secretion into the supernatant was confirmed by Western blotting. The supernatant of pCrl-transfected cells showed a major Reelin band at approximately 400 kDa, and minor bands at 350 and 140 kDa (Fig. 2*B*, *lane 2*). The supernatant of mock-transfected cells did not show any

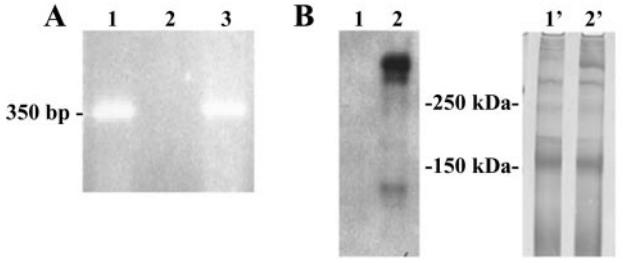

FIG. 2. **HEK 293T cells transfected with the pCrl vector express Reelin mRNA and secrete Reelin in the supernatant.** *A,* total RNA was extracted from cell homogenates and pCrl mRNA expression was probed with RT-PCR, using primers corresponding to exon 27 of Reelin. *Lanes 1*, PCR of pCrl vector; *2*, RT-PCR of mock-(pCDNA3)-transfected cells; *3*, RT-PCR of pCrl-transfected cells. The position of the 350-bp marker is indicated on the *left*. *B,* Western blot of cell culture supernatants, probed with anti-Reelin mAb 142. *Lanes 1*, supernatant from mock-transfected cells; *2*, supernatant from pCrl-transfected cells; 1′ and 2′ replica samples of *lanes 1* and *2*, after silver stain. The position of 250- and 150-kDa markers is indicated.

stained bands (*lane 1*).

HEK 293T cells endogenously express $\alpha_5\beta_1$ integrin which specifically mediates adhesion to fibronectin (28). Titration experiments showed dose-dependent adhesion to fibronectin, with maximal adhesion occurring at 2.5–5 μg/ml fibronectin (data not shown). Reelin-transfected HEK 293T showed significantly less adhesion as compared with mock-transfected HEK 293T cells. After a 2-h incubation, 44.7 ± 6.3 (mean ± S.E.) pCrl-transfected cells were attached to the substrate, as compared with 96.3 ± 10.8 mock-transfected cells (Fig. 3*A*, $p < 0.001$). Furthermore, cell morphology was markedly different: Reelin-secreting cells appeared unable to spread on fibronectin and their processes were diminished in number and length, as compared with mock-transfected cells (Fig. 3*B*). To assess the biological significance of the enzymatic activity of Reelin, the effect of DFP, a potent and specific inhibitor of serine hydrolases, on cell adhesion was studied. Micromolar concentrations of DFP partially restored adhesion of Reelin-expressing cells on fibronectin, without affecting mock-transfected cells (Fig. 3, *A* and *B*). The effect of DFP was dose-dependent, starting at concentrations ≥0.54 μM; maximal increase in adhesion of Reelin-expressing cells was seen at 5.4 μM DFP, while 5.4 mM was equally toxic for pCrl- and mock-transfected cells, inhibiting adhesion of >99% of the cells (Fig. 3*C*).

Reelin Binds a Serine Hydrolase Probe—FP-Peg-biotin is described to behave as a specific and irreversible probe for serine hydrolases, showing properties similar to those of common FP inhibitors, such as DFP (30). To explore Reelin labeling with FP-Peg-biotin, aliquots of transfected 293T cell culture supernatants incubated with 5 μM FP-Peg-biotin were separated on standard SDS-PAGE gels, blotted, and probed with avidin peroxidase; replica samples were stained with the monoclonal antibody 142. The supernatant of Reelin-expressing cells showed distinct Reelin bands at approximately 400, 300, and 140 kDa (Fig. 4*A*, *lane 2*). The 400- and 300-kDa band showed faint labeling with FP-Peg-biotin, while the 140-kDa band showed strong labeling with FP-Peg-biotin (Fig. 4*B*, *lane 1*; *arrows* indicate corresponding bands in the two blots). Labeling of these three bands was inhibited by DFP (Fig. 4*B*, *lane 2*). The supernatant of mock-transfected cells showed a completely different labeling pattern with FP-Peg-biotin: the most evidently labeled band was a 150-kDa band, while no labeled bands were visible at 400 and 140 kDa (Fig. 4*B*, *lane 3*). Given the complex pattern of labeling with FP-Peg-biotin in supernatants, we decided to perform FP-Peg-biotin labeling on partially purified Reelin. The supernatant of the stable cell line CER, expressing high levels of Reelin, was concentrated and partially purified

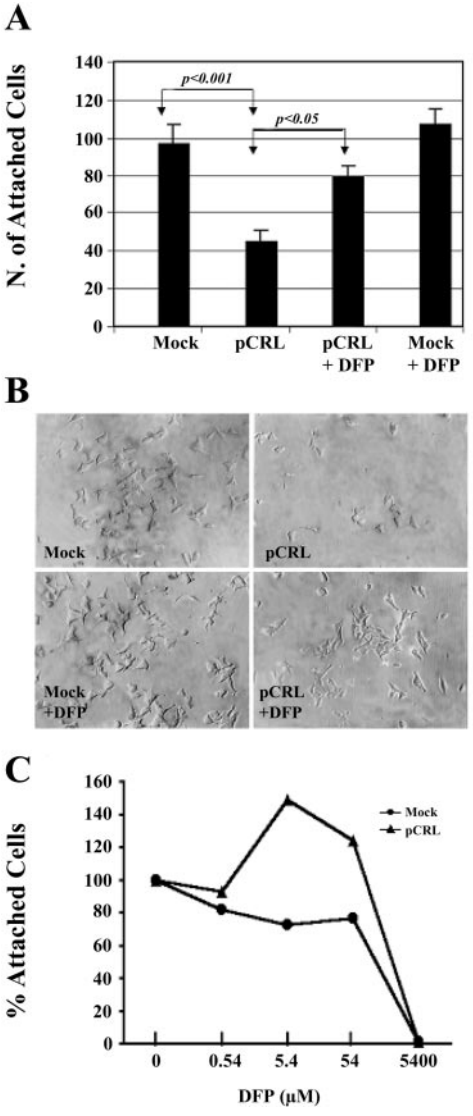

FIG. 3. **Expression of Reelin inhibits adhesion of HEK 293T cells to fibronectin, and DFP treatment reverses the effect of Reelin expression.** *A,* quantification of the effect of various treatments. *Bars* show the numbers of cells attached to fibronectin-coated wells under different conditions. Each *bar* represent the mean and S.E. of five wells. *Double-headed arrows* indicate the statistical difference between groups (one-way ANOVA followed by LSD post-hoc test). *B,* phase-contrast images of cells grown in different conditions. DFP was applied at a concentration of 5.4 μM. *C,* dose-dependent effect of DFP on cell adhesion of mock- or pCrl-transfected HEK 293T cells. Each point represents the percent ratio between the number of attached cells after DFP treatment and the number of attached cells in the absence of DFP.

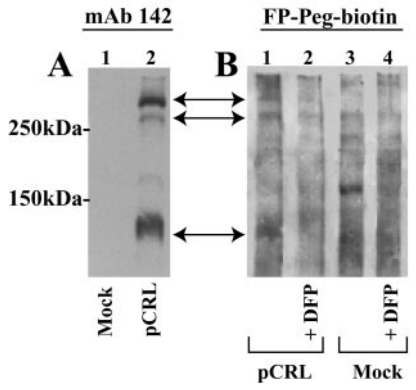

FIG. 4. **Reelin can be labeled with FP-Peg-biotin, a serine trap probe, and labeling is inhibited by DFP.** *A,* aliquots of supernatants were separated by SDS-PAGE, blotted, and stained with mAb 142. *Lanes 1*, Mock-transfected HEK 293T cells; *2*, pCrl-transfected cells; *arrows* indicate Reelin bands. *B,* FP-Peg-biotin labels several bands in the supernatants. *Lanes 1*, supernatant of pCrl-transfected cells; *arrows* indicate bands corresponding to the 400-, 300-, and 140-kDa Reelin bands; *2*, replica sample as in *lane 1*, but preincubated with 11 μM DFP; *3*, supernatant of mock-transfected cells; *4*, replica sample as in *lane 3*, but preincubated in the presence of 11 μM DFP. Two-hundred and fifty ng of total protein were applied to each lane.

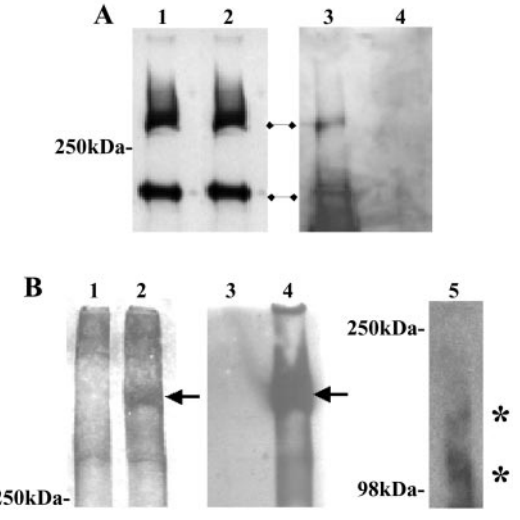

FIG. 5. **Purification of Reelin by fast protein liquid chromatography and SDS-PAGE electroelution, and labeling of purified Reelin with FP-Peg-biotin.** *A,* the concentrated supernatant from CER cells was purified on a Sephadex-200 gel filtration column; the Reelin-positive eluate from the column was separated on a 4–12% gradient SDS-PAGE. *Lanes 1* and *2*, immunoblot (mAb E4) of the sample incubated with FP-Peg-biotin after pretreatment without and with PMSF, respectively. *Lanes 3* and *4*, the blot was stripped and developed with horseradish peroxidase-conjugated streptavidin to reveal bound FP-Peg-biotin. The 250-kDa calibration marker shown next to *lane 1* is valid for all 4 lanes. *B,* supernatants of mock-transfected (*lane 1*) and pCrl-transfected cells (*lane 2*) were separated with SDS-PAGE (5% gel) for 3 h 30 min and the gel was stained with silver nitrate. The *arrow* next to *lane 2* indicates the 400-kDa Reelin band. *Lanes 3* and *4*, blot corresponding to *lanes 1* and *2*, respectively, stained with anti-Reelin mAb 142. The *arrow* next to *lane 4* points to 400-kDa Reelin. The 250-kDa calibration marker next to *lane 1* is valid for *lanes 2–4* as well. *Lane 5*, the 400-kDa Reelin band shown in *lane 2* was electroeluted, electrophoresed in a second gel, blotted, and stained with mAb 142. The *asterisks* mark degradation products of Reelin at approximately 180 and 140 kDa. The 400-kDa band has practically disappeared.

by gel filtration chromatography. The Reelin-positive eluate from the Superdex 200 gel filtration column was concentrated and then incubated in the absence or presence of the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF). The samples were separated by SDS-PAGE on a 4–12% gradient gel, blotted, and incubated with the monoclonal antibody E4 to reveal Reelin (Fig. 5*A*, *lanes 1* and *2*)**.** The blot was then stripped and incubated with streptavidin to reveal the binding to FP-Peg-biotin (Fig. 5*A*, *lanes 3* and *4*). As with the crude supernatant (Figs. 2 and 4), the immunoblot showed two major Reelin-positive bands, one higher than the 250-kDa marker, corresponding to the 400- and 300-kDa isoforms, and a smaller band at about 140 kDa (Fig. 5*A*, *lanes 1* and *2*). FP-Peg-biotin binds to both major Reelin bands in the absence, but not in the presence of PMSF (Fig. 5*A*, *lanes 3* and *4*). The smaller band appeared to bind FP-Peg-biotin with a higher affinity than the higher molecular weight isoform (Fig. 5*A*, *lane 3*).

Reelin Shows Protease Activity on Extracellular Matrix Proteins in Vitro—To investigate the catalytic activity of Reelin, we first further purified the high molecular weight isoforms by SDS-PAGE and electroelution. As shown in Fig. 5*B*, separation

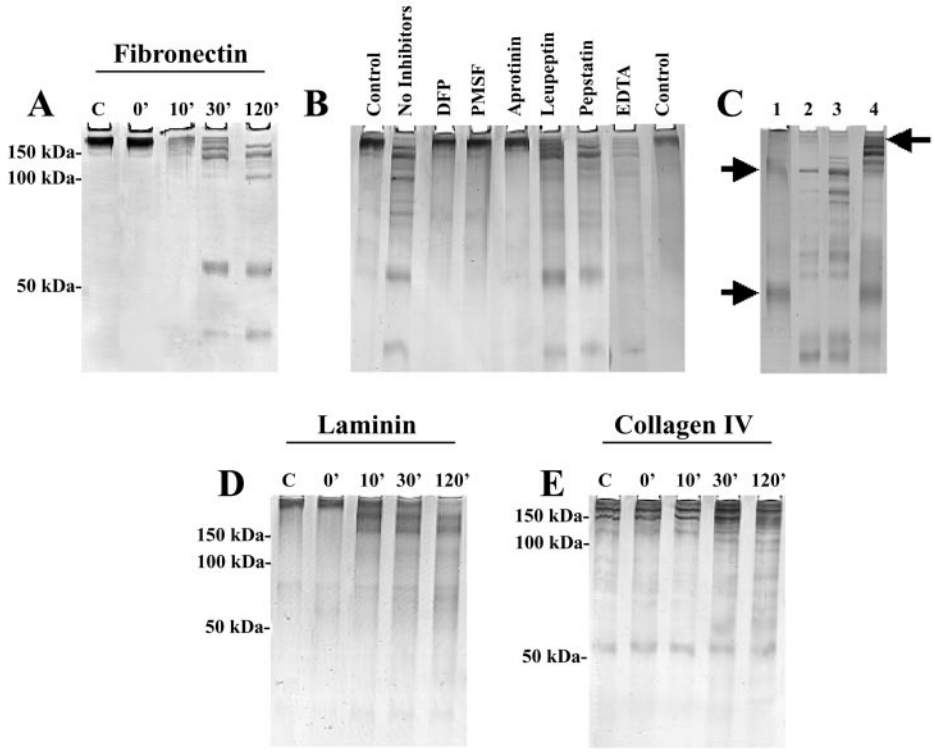

FIG. 6. **Purified Reelin rapidly degrades ECM proteins fibronectin and laminin, while collagen IV is degraded at a much slower rate.** Protein degradation was assessed with SDS-PAGE and silver staining of gels. *A,* time course of fibronectin degradation by Reelin; human fibronectin (1 μg) was incubated with purified Reelin (5 ng) for 0, 10, 30, and 120 min at 37 °C. C: intact fibronectin. *B,* degradation of fibronectin by Reelin is inhibited by serine protease inhibitors, but not by inhibitors of other classes of proteases; fibronectin was incubated with Reelin for 120 min at 37 °C in the presence of different protease inhibitors; control, intact fibronectin; concentrations of inhibitors are: DFP, 21.6 mM; PMSF, 2 mg/ml; aprotinin, 1 mg/ml; leupeptin, 1 mg/ml; pepstatin, 0.1 mg/ml; EDTA, 10 mM. *C,* degradation of fibronectin by Reelin is partially inhibited by mAb CR-50. *Lane 1,* purified CR-50, *arrows* point to the heavy and light chains of the antibody. *Lanes 2–4*, fibronectin incubated with Reelin for 120 min at 37 °C in the absence (*lane 2*) or presence (*lanes 3* and 4) of mAb CR-50 (*lane 3*, 0.098 μg/ml; *lane 4*, 9.8 μg/ml). *Arrow* next to *lane 4* points to intact fibronectin. *D,* degradation of laminin by Reelin; *C*, laminin alone. *E,* degradation of collagen IV by Reelin; *C*, collagen IV alone. The positions of molecular weight markers are indicated on the *left* of the gels. Molecular weight markers indicated in *A* are also for *B* and *C*.

of the concentrated cell culture supernatant using a 5% SDS gel resulted in a good separation of the Reelin isoforms. Silver nitrate staining indicated that only the supernatant of Reelin expressing cells contains a band at approximately 400 kDa corresponding to the Reelin isoforms of 400 (and 300) kDa (Fig. 5*B*, *lanes 2* and *4*). Therefore, the high molecular weight Reelin band was electroeluted from the gel to achieve a high degree of purification. The electroeluted sample was reanalyzed by SDS-PAGE and Western blotting (Fig. 5*B*, *lane 5*). However, we found that the purified high molecular weight Reelin protein quickly disappeared and smaller bands appeared around 180 and 140 kDa, probably corresponding to self-degradation products. The major proteolytic product of 140 kDa that we observed in this study may correspond to the 180-kDa degradation product that has been described by other investigators (8, 35).

To test for proteolytic activity on extracellular matrix proteins, purified Reelin was incubated with pure fibronectin, laminin, or collagen IV, and breakdown products were analyzed by SDS-PAGE and silver staining of gels. Fibronectin and laminin breakdown fragments were seen already after 10 min incubation (Fig. 6, *A* and *D*), while collagen IV was degraded at a much slower rate (Fig. 6*E*). Fibronectin degradation was blocked by inhibitors of serine proteases (DFP, PMSF, and aprotinin), but not by inhibitors of other families of proteases (Fig. 6*B*). Fibronectin degradation was also partially inhibited by monoclonal antibody CR-50 (Fig. 6*C*), an antibody directed against the NH_2-terminal portion of Reelin that has been demonstrated to inhibit Reelin function both *in vitro* and *in vivo* (see Ref. 31 and references quoted therein). Inhibition of fibronectin degradation was seen at a CR-50 concentration of 9.8 μg/ml, the highest concentration tested. Interestingly, this concentration is comparable with the concentrations that have been reported in the literature to inhibit Reelin function (20–200 μg/ml, Ref. 31).

DISCUSSION

In this paper we present converging biochemical and cellular evidence that Reelin is a serine protease of the extracellular matrix, and that its enzymatic activity is important for the modulation of cell adhesion. The fact that CR-50, a monoclonal antibody known to inhibit Reelin function both *in vitro* and *in vivo*, blocks the proteolytic activity of Reelin further supports the hypothesis that proteolytic activity is of fundamental importance for the function of Reelin. These findings appear interesting in view of the fact that serine proteases, such as tissue plasminogen activator (tPA), are already known to be important modulators of cell migration and axon growth (32).

HEK 293T cells adhere quickly to fibronectin, due to endogenous expression of $\alpha_5\beta_1$ integrin (28), which is a selective fibronectin receptor (33). In this paper we show that expression of Reelin leads to a marked decrease of adhesion of HEK 293T cells to fibronectin. These data, together with the demonstration that purified Reelin degrades fibronectin *in vitro*, are consistent with the hypothesis that Reelin, secreted by HEK 293T cells, inhibits cell adhesion by degrading the fibronectin substrate. Alternatively, Reelin might activate other targets, for example, cell membrane receptors or other proteases, which are in turn directly responsible for cell detachment. A third hypothesis is that Reelin induces the expression of another

serine protease, which is in turn responsible for cell detachment. This hypothesis appears remote, since we have demonstrated that Reelin binds FP-Peg-biotin, and degrades fibronectin *in vitro*.

We found that, after purification, Reelin appears to undergo rapid self-degradation. Our data suggest that the major 140-kDa fragment is enzymatically active, since its binding to FP-Peg-biotin is even stronger than that of full-length Reelin. Interestingly, we also observed strong labeling with FP-Peg-biotin of the smaller fragment after immunoaffinity purification of Reelin from mouse brain.[2] These data support the idea that the proteolytic processing of Reelin is functionally important, and that full activity of Reelin might require degradation of the 400-kDa full-length precursor to generate smaller, more active isoforms.

Reelin appears to behave as a specific serine protease, as collagen IV is degraded at a much slower rate than fibronectin or laminin. However, this hypothesis needs further confirmation, using model peptide substrates.

Reelin has been suggested to allow migrating neurons to grow past previously migrated cells and to promote detachment of neurons from radial glial fibers (6–8). The α_5 integrin subunit, expressed in HEK 293T cells, shows high homology with the α_3 subunit, which is expressed on migrating neurons, and appears to be involved in the inhibitory effect of Reelin on neuronal migration along radial glial processes. Reelin has been demonstrated to bind to $\alpha_3\beta_1$ integrin (8). *In situ* hybridization experiments and double immunolabeling with antibodies against fibronectin and antibodies against radial glia demonstrate transient fibronectin expression on radial glia processes during early stages of cortical development, until completion of corticogenesis (34). On the basis of the available evidence, we propose that $\alpha_3\beta_1$ integrin might immobilize extracellular Reelin on the surface of migrating neurons and thus focus its proteolytic activity on fibronectin expressed on radial glial cells. Alternatively, binding to $\alpha_3\beta_1$ integrin might enhance the activity of Reelin by protecting it from degradation. In this respect it is interesting to notice that elevated levels of cleaved Reelin have been detected in the absence of $\alpha_3\beta_1$ integrin. This finding has been interpreted as evidence that $\alpha_3\beta_1$ integrin inhibits degradation of Reelin by modulating the activity of a zinc-dependent metalloproteinase (8, 35). Our data are consistent with the simpler hypothesis that the appearance of Reelin fragments is the result of a self-degradation activity, and that $\alpha_3\beta_1$ integrin might protect Reelin from self-degradation.

Our data are consistent with a role for integrins in Reelin-mediated cell adhesion. However, the action of Reelin on neuronal migration may be mediated by other pathways, such as the very low density lipoprotein receptor/apoE receptor 2/disabled-1 pathway (17–19). It is conceivable, for example, that Reelin may cleave these lipoprotein receptors and thereby activate the disabled-1 signaling pathway. Furthermore, in our cell culture model the action of Reelin is incompletely blocked by DFP, indicating that Reelin might also act via pathways that are independent of serine protease activity. Finally, it should be noted that $\alpha_5\beta_1$, the integrin expressed by HEK 293T cells, is a highly selective receptor for fibronectin (33). Thus, HEK 293T cells might be much more sensitive to integrin-fibronectin interactions than cortical neurons in the developing brain, where other cell adhesion complexes might be predominant.

The discovery of Reelin's function as a serine protease of the extracellular matrix is also intriguing when considering the expression and distribution of Reelin and adhesion molecules of the extracellular matrix in structures other than the developing cerebral cortex. Reelin is co-expressed with integrins in dendritic spines of GABAergic neurons in the adult cerebral cortex, and of glutamatergic neurons in the cerebellum and olfactory bulb (10, 11, 36). Dendritic spine density is decreased in fronto-parietal cortex and CA1 pyramidal neurons of heterozygous *reeler* mice (37), and heterozygous *reeler* mice show abnormalities in complex behavior, like neophobia and increased anxiety (38), a finding that led investigators to propose these mice as an animal model of schizophrenia. We suggest that the synaptic role of Reelin as a serine protease might consist in a rapid and local modulation of adhesive forces between pre- and post-synaptic elements, thus modulating the efficiency of synaptic transmission at the local level. In fact, other serine proteases, like tPA and plasmin, have already been suspected to be involved in synaptic plasticity. tPA contributes to the late phase of long-term potentiation in hippocampal slices and stimulates synapse formation in hippocampal cell culture (39). Plasmin cleaves laminin and appears to regulate long-term potentiation (40). In conclusion, our findings may help to better understand the roles of Reelin both in physiology and in disease, by bringing this protein into the complex and exciting scenario of protease-regulated signaling networks.

Acknowledgments—We are deeply grateful to Dr. A. M. Goffinet for the gift of monoclonal antibodies 142 and G10 and Dr. Benjamin Cravatt for the gift of FP-Peg-biotin. Furthermore, we thank Graziano Bonelli, Marco De Luca, and Ramona Marino for technical assistance, and Flavia Mancuso for editorial assistance.

REFERENCES

1. D'Arcangelo, G., Miao, G. G., Chen, S., Soares, H. D., Morgan, J. I. & Curran, T. (1995) *Nature* **374,** 719–723
2. De Silva, U., D'Arcangelo, G., Braden, V. V., Chen, J., Miao, G. G., Curran, T. & Green, E. D. (1997) *Genome Res.* **7,** 157–164
3. Goffinet, A. M. (1984) *Brain Res. Brain Res. Rev.* **7,** 261–296
4. Alcantara, S., Ruiz, M., D'Arcangelo, G., Ezan, F., de Lecea, L., Curran, T., Stelo, C. & Soriano, E. (1998) *J. Neurosci.* **18,** 7779–7799
5. D'Arcangelo, G., Nakajima, K., Miyata, T., Ogawa, M., Mikoshiba, K. & Curran, T. (1997) *J. Neurosci.* **17,** 23–31
6. Pinto-Lord, M. C., Evrard, P. & Caviness, V. S. (1982) *Brain Res.* **256,** 379–393
7. Hoffarth, R. M., Johnston, J. G., Krushel, L. A. & van der Kooy, D. (1995) *J. Neurosci.* **15,** 4838–4850
8. Dulabon, L., Olson, E. C., Taglienti, M. G., Eisenhuth, S., McGrath, B., Walsh, C. A., Kreidberg, J. A. & Anton, E. S. (2000) *Neuron* **27,** 33–44
9. Anton, E. S., Kreidberg, J. A. & Rakic, P. (1999) *Neuron* **22,** 277–289
10. Pesold, C., Liu, W. S., Guidotti, A., Costa, E. & Caruncho, H. J. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 3217–3222
11. Pesold, C., Impagnatiello, F., Pisu, M. G., Uzunov, D. P., Costa, E., Guidotti, A. & Caruncho, H. J. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 3221–3226
12. Smalheiser, N. R., Costa, E., Guidotti, A., Impagnatiello, F., Auta, J., Lacor, P., Kriho, V. & Pappas G. D. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 1281–1286
13. Hong, S. E., Shugart, Y. Y., Huang, D. T., Shahwan, S., Grant, P. E., Hourihane, J. O. B., Martin, N. D. T. & Walsh, C. A. (2000) *Nat. Genet.* **26,** 93–96
14. Guidotti, A., Auta, J., Davis, J. M., Gerevini, V. D., Dwivedi, Y., Grayson, D. R., Impagnatiello, F., Pandey, G., Pesold, C., Sharma, R., Uzunov, D. & Costa, E. (2000) *Arch. Gen. Psychiatry* **57,** 1061–1069
15. Persico, A. M., D'Agruma, L., Maiorano, N., Totaro, A., Militerni, R., Bravaccio, C., Wassink, T. H., Schneider, C., Melmed, R., Trillo, S., Montecchi, F., Palermo, M., Pascucci, T., Puglisi-Allegra, S., Reichelt, K. L., Conciatori, M., Marino, R., Quattrocchi, C. C., Baldi, A., Zelante, L., Gasparini, P. & Keller, F. (2001) *Mol. Psychiatry* **6,** 150–159
16. Klar, A., Baldassare, M. & Jessell, T. M. (1992) *Cell* **69,** 95–110
17. Hiesberger, T., Trommsdorff, M., Howell, B. W., Goffinet, A., Mumby, M. C., Cooper, J. A. & Herz, J. (1999) *Neuron* **24,** 481–499
18. D'Arcangelo, G., Homayouni, R., Keshvara, L., Rice, D. S., Sheldon, M. & Curran, T. (1999) *Neuron* **24,** 471–479
19. Trommsdorff, M., Gotthardt, M., Hiesberger, T., Shelton, J., Stockinger, W., Nimpf, J., Hammer, R. E., Richardson, J. A. & Herz, J. (1999) *Cell* **97,** 689–701
20. Senzaki, K., Ogawa, M. & Yagi, T. (1999) *Cell* **99,** 635–647
21. Kalafatis, M., Egan, J. O., van't Veer, C., Cawther, K. M. & Mann, K. G. (1997) *Crit. Rev. Eukaryotic Gene Expr.* **7,** 241–280
22. Kinoshita, H., Sakiyama, H., Tokunaga, K., Imajoh-Ohmi, S., Hamada, Y., Isono, K. & Sakiyama, S. (1989) *FEBS Lett.* **250,** 411–415
23. Kusumoto, H., Hirosawa, S., Salier, J. P., Hagen, F. S. & Kurachi, K. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 7307–7311
24. Cooper, J. A. & Howell, B. W. (1999) *Cell* **97,** 671–674

[2] C. C. Quattrocchi and F. Keller, unpublished observations.

25. Gliemann, J. (1998) *Biol. Chem.* **379,** 951–964
26. Chomczynski, P. & Sacchi N. (1987) *Anal. Biochem.* **162,** 156–159
27. de Bergeyck, V., Naerhuyzen, B., Goffinet, A. M. & Lambert de Rouvroit, C. (1998) *J. Neurosci. Methods* **82,** 17–24
28. Abraham, D. G., Nutt, E. M., Bednar, R. A., Bednar, B., Gould, R. J. & Duong, Le T. (1997) *Mol. Pharmacol.* **52,** 227–236
29. Laemmli, U. K. (1970) *Nature* **227,** 680–685
30. Liu, Y., Patricelli, M. P. & Cravatt, B. F. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 14694–14699
31. Utsunomiya-Tate, N., Kubo, K., Tate, S., Kainosho, M., Katayama, E., Nakajima, K. & Mikoshiba, K. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 9729–9734
32. Seeds, N. W., Basham, M. E. & Haffke, S. P. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 14118–14123
33. Miyamoto, S., Katz, B. Z., Lafrenie, R. M. & Yamada, K. M. (1998) *Ann. N. Y. Acad. Sci.* **857,** 119–129
34. Sheppard, A. M., Hamilton, S. K. & Pearlman, A. L. (1991) *J. Neurosci.* **11,** 3928–3942
35. Lambert de Rouvroit, C., de Bergeyck, V., Cortvrindt, C., Bar, I., Eeckhout, Y. & Goffinet, A. M. (1999) *Exp. Neurol.* **156,** 214–217
36. Rodriguez, M. A., Pesold, C., Liu, W. S., Kriho, V., Guidotti, A., Pappas, G. D. & Costa, E. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 3550–3555
37. Liu, W. S., Pesold, C., Rodriguez, M. A., Carboni, G., Auta, J., Lacor, P., Larson, J., Condie, B. G., Guidotti, A. & Costa, E. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 3477–3482
38. Tueting, P., Costa, E., Dwivedi, Y., Guidotti, A., Impagnatiello, F., Manev, R. & Pesold, C. (1999) *Neuroreport* **10,** 1329–1334
39. Baranes, D., Lederfein, D., Huang, Y., Chen, M., Bailey, C. H. & Kandel, E. (1998) *Neuron* **21,** 813–825
40. Nakagami, Y., Abe, K., Nishiyama, N. & Matsuki, N. (2000) *J. Neurosci.* **20,** 2003–2010