Amyloid Precursor Protein and Presenilin1 Interact with the Adaptor GRB2 and Modulate ERK1,2 Signaling

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The amyloid precursor protein (APP) and the presenilins 1 and 2 are genetically linked to the development of familial Alzheimer disease. APP is a single-pass transmembrane protein and precursor of fibrillar and toxic amyloid-β peptides, which are considered responsible for Alzheimer disease neurodegeneration. Presenilins are multipass membrane proteins, involved in the enzymatic cleavage of APP and other signaling receptors and transducers. The role of APP and presenilins in Alzheimer disease development seems to be related to the formation of amyloid-β peptides; however, their physiological function, reciprocal interaction, and molecular mechanisms leading to neurodegeneration are unclear. APP and presenilins are also involved in multiple interactions with intracellular proteins, the significance of which is under investigation. Among the different APP-interacting proteins, we focused our interest on the GRB2 adaptor protein, which connects cell surface receptors to intracellular signaling pathways. In this study we provide evidence by co-immunoprecipitation experiments, confocal and electron microscopy, and by fluorescence resonance energy transfer experiments that both APP and presenilin1 interact with GRB2 in vesicular structures at the centrosome of the cell. The final target for these interactions is ERK1,2, which is activated in mitotic centrosomes in a PS1- and APP-dependent manner. These data suggest that both APP and presenilin1 can be part of a common signaling pathway that regulates ERK1,2 and the cell cycle.

Alzheimer disease is a heterogeneous neurodegenerative disorder with insidious onset and irreversible progression, genetically linked to few molecules as follows: APP on chromosome 21 and the two presenilins (PSs) on chromosome 14 (PS1) and 1 (PS2), respectively (1). The molecular mechanisms causing sporadic and familial (FAD) forms of AD are not yet known, and the physiological functions of APP and PSs are also unclear. APP is a type 1 transmembrane protein whose proteolytic processing generates long soluble N-terminal fragments, a family of short soluble amyloid-β peptides (Aβ) (2, 3), and a second intracellular C-terminal fragment named AICD from “APP intracellular domain,” which is likely involved in gene regulation (4, 5). Aβ peptides, as water-soluble oligomers or insoluble aggregates, are toxic species suspected to cause neurodegeneration and synaptic loss that, together with reactive gliosis and intracellular tangles of hyperphosphorylated Tau protein, represent the classical neuropathological hallmarks of the disease (6). PSs are multitransmembrane proteins, which may accumulate as endoproteolyzed heterodimers of N- and C-terminal fragments associated with other membrane proteins (i.e. nicastrin, APH-1, and PEN-2) to form high molecular weight complexes (7) responsible for the intramembranous γ-secretase cleavage of APP, low density lipoprotein receptor-related protein, E-cadherin, Notch-1, CD44, and ErbB4 (8–12). The hypothesized role of PS mutations in the genesis of FAD is apparently linked to the enhanced APP cleavage and formation of Aβ-(x-42) isoforms, increasing the activity of the multiproteic γ-secretase complex (6).

If we consider that there is a significant phenotypical heterogeneity among patients, and even among familial patients bearing the same genetic mutation, it seems likely that other proteins modulate APP and PS functions. In fact, APP and PSs

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The abbreviations used are: APP, amyloid precursor protein; AD, Alzheimer disease; ERK, extracellular signal-regulated kinase; MEF, mouse embryo fibroblast; FRET, fluorescent resonance energy transfer; Tricine, N-(2-hydroxy-1,1-bis[(hydroxymethyl)methyl]glycine; PBS, phosphate-buffered saline; Pipes, 1,4-piperazinediethanesulfonic acid; GST, glutathione S-transferase; siRNA, short interfering RNA; PS, presenilin; FAD, familial AD; SH, Src homology; RT, reverse transcriptase; IP, immunoprecipitation; CTF, C-terminal fragment; EGFP, enhanced green fluorescent protein; Aβ, amyloid-β peptides; MEK, MAPK/ERK kinase; ER, endoplasmic reticulum; APLP, APP-like protein.
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are in the center of a network of protein-protein interactions whose significance for the regulation of Aβ formation and generally for AD development is under extensive investigation (13). In addition to their proteolytic roles culminating in Aβ production, PSs have been shown to interact with various proteins involved in the regulation of γ-secretase activity, cell survival, development, and signaling. In particular, PSs are implicated in Notch and Wnt signaling, cell-cell adhesion, vesicular transport, apoptosis, calcium signaling, phosphorylation and degradation of β-catenin, modulation of ERK1,2, and phosphatidylinositol 3-kinase activity (13–17). On the other side, the APP C-terminal domain is recognized by a plethora of adaptors and signaling molecules, the role of which for AD development is still unclear (18, 19).

Among the different APP-interacting proteins, we focused our interest on the GRB2 (growth factor receptor-bound protein 2) adaptor protein, which usually provides a critical link between cell surface growth factor receptors, Ras signaling, and cell proliferation (20, 21), and whose interaction with APP is enhanced in AD brain (22). Besides its involvement in signal transduction pathways mediated by tyrosine kinase receptors, GRB2 may also anchor to a number of proteins involved in cell signaling and vesicular trafficking, such as dynamin and synapsin (23, 24), or to proteins regulating cytoskeletal dynamics, cell cycle, and metastatic proliferation (21, 25–27). GRB2 is composed of a central SH2 domain flanked by two SH3 domains and interacts through its SH2 domain with the C-terminal 682YENPTY687 motif of APP upon the specific phosphorylation of Tyr-682 (numbering on APP695 isoform) (28, 29). At present, it is unclear whether APP may have a role in some of the cell activities in which GRB2 participates or whether GRB2 modulates or affects APP function or even amyloid formation.

Here we provide evidence that GRB2 interacts with APP and PS1 in vesicular structures dispersed in the cytosol and mainly in the pericentriolar material at the centrosome, a crucial region for microtubule nucleation, cell cycle progression, migration, cytokinesis, and cellularization (30). We also provide evidence that APP and PS1 modulates the centrosomal translocation or phosphorylation of ERK1,2 during mitosis and that the activation of ERK1,2 depends on phosphorylation of tyrosine 682 of APP and on APP cleavage. These findings therefore raise the possibility that perturbations in MEK/ERK signal- ing may constitute a common metabolic pathway modulated by both gene products that cause familial AD.

EXPERIMENTAL PROCEDURES

Recombinant Constructs—APP695 wild type, APPY653F, APPY682A, Y687A, and Y682A/Y687A mutants have been described previously (31). The APP-EGFP construct was made as follows. The human APP695 cloned into the pRc/CMV vector (Invitrogen), as described previously, was the template for mutagenesis reaction (QuikChange® mutagenesis kit, Stratagene, La Jolla, CA). A set of primers, 5'-GAGCAGATGCAA-CCGGTCCCCCGCCACACGC-3' sense and 5'-GCTCAAGA-ACCTTGGCGTTGGATTTCG-3' antisense, were designed to add the AgeI site and remove the stop codon to the 3' end of APP. The mutated DNA was digested and ligated into the SacII and AgeI site of the pEGFP-N1 vector (Clontech). Human PS1 DNA was cloned into the HindIII, XhoI site of the expression vector pcDNA3.1-V5 (Invitrogen). The PS1 mutants H163R and L286V were prepared by mutagenesis reaction (Stratagene) using pcDNA3.1-PS1-V5 (Invitrogen) as template and the following specific primers: H163R sense 5'-GGTGGCTTAAAG-GTCATCGTGCGTGGGTATTATTA-3' and H163R antisense 5'-TAATAAGCCAGGAacGATGACCTTATAGACCC-3' ; L286V sense 5'-CGGTATTTCGTCGTTAATTCCTCC-TCAAC-3' and L286V antisense 5'-GTGAGGATTAATTGAcAGCTGGAAAAACGC-3'. BACE1 cDNA was kindly provided by Dr. R. Nitsch and was cloned into the pcDNA3.1 vector. Human GRB2 gene was cloned into the BamHI/XbaI site of the expression vector pcDNA3.1-V5/His (Invitrogen) to obtain the histidine tag. To get a fusion protein between GRB2 and glutathione S-transferase (GRB2-GST), the human GRB2 cDNA was cloned into the EcoRI/AgeI site of the expression vector pGEX-4T-2 (GE Healthcare). siRNAs corresponding to β-secretase BACE1 was done as described previously (32), with minor modifications, using Silencer siRNA construction kit (Ambion Inc. Austin, TX). The following set of oligonucleotides was designed: siRNA1 sense 5'-CATCCTGTTGGATA-CAGGC-3' and antisense 5'-GCCGTATACCAAGCGTG-3'; siRNA2 sense 5'-TGACGTAGAGGATACACA-3' and antisense 5'-TTGAATCTCTTCGACAGGC-3'; siRNA3 sense 5'-TGGCTTTGGCTGTCAGCGC-3' and antisense 5'-GGACTGGGACAGGCACCGG-3'. Results obtained from siRNA2 are shown. RT-PCR was done in H4 wild type cells and H4-BACE1 transfected cells grown to confluence, and total RNA was isolated using the RNeasy mini kit from Qiangen (Valencia, CA). Two micrograms of total RNA were subjected to reverse transcriptase (RT) first strand synthesis using the Superscript kit (Invitrogen) according to the manufacturer’s instructions. Equal amounts of the RT product were then used for PCR of BACE1 or BACE2 using specific primers.

Antibodies—Polyclonal antibodies 13-0200 and 51-2700, respectively, for the N and C terminus of APP were from Zymed Laboratories Inc. and were used at a dilution of 1:150 for immunoprecipitation and 1:1,000 for immunodetection. The antibody C-20 for the C terminus of APP was from Santa Cruz Biotechnology (Santa Cruz, CA), and the rabbit polyclonal antibody for the N terminus of APP was from Sigma and was used in immunofluorescence at a 1:100 dilution. The antibody R3659 specific to the N terminus of Aβ and C99 was a gift from Dr. P. Gambetti (Case Western Reserve University, Cleveland, OH) was used 1:100 in immunoprecipitation. Antibodies for GRB2 used in Western blotting (1:5000) or in immunofluorescence (1:100) were from BD Transduction Laboratories and Santa Cruz Biotechnology (C-23 and E-1), respectively. Anti-ShcA antibody (Upstate Biotechnology, Inc., Lake Placid, NY) was used at 1:200 for immunoprecipitation. The phospho-p44/42 ERK and unphosphorylated p44/42 ERK antibodies from Cell Signaling Technology (Danvers, MA) were used at 1:1,000 and 1:100, respectively, for immunoblotting and immunofluorescence experiments. PS1 N-terminal antibody from Calbiochem was used 1:100 in immunoprecipitation. The polyclonal antibody against γ-tubulin (Sigma) and pericentrin-specific antibody (Abcam Cam-
bridge, UK) were used at 1:200 and 1:500, respectively, in immunofluorescence experiments. Antibody anti-Golgin97 specific for the Golgi compartment was used at 1:2,000 in immunoblotting (Invitrogen). Anti-V5 antibody (Invitrogen) and anti-FLAG M2 monoclonal antibody (Sigma) were used at 1:100 in immunoprecipitation. Polyclonal antibody F25608 antibody specific for the C terminus of APP and CTFs (kindly provided by Dr. P. Gambetti, Case Western Reserve University, Cleveland, OH) was used at 1:100 for immunoprecipitation. The polyclonal antibody anti-BACE1 (custom made) recognizing the C-terminal region (amino acids 486–501) was used at 1:1,000 for immunodetection. Anti-CD26 antibody (Santa Cruz Biotechnology) was used at 1:100 for immunoprecipitation. Alexa Fluor® 568 and Alexa Fluor® 488 (Invitrogen) were used 1:250 for immunofluorescence. Anti-VHIs antibody conjugated with Alexa 568 (Invitrogen) was used at 1:100 for immunofluorescence. Substances and specific drugs used in this paper were from Sigma.

**Cell Cultures**—H4 cells and H4 cells stably expressing BACE1 with a C-terminal FLAG tag were kindly provided by Prof. Roger M. Nitsch (University of Zurich) and maintained in the presence of 500 μg/ml G418. MEF APP/APLPs null cells were kindly provided by Dr. U. Mueller, and MEF cells wild type and PS1(−/−) (genetically deficient in PS1 and PS2) have been described previously (33) and were provided by Dr. B. De Strooper. All cell lines were grown in Dulbecco’s modified Eagle’s medium (EuroClone, Paignton-Devon, UK) supplemented with 10% fetal bovine serum (Invitrogen), L-glutamine (EuroClone, Paignton-Devon, UK), and antibiotics (penicillin and streptomycin) (EuroClone, Paignton-Devon, UK), unless explicitly stated otherwise. Transient transfections were carried out using FuGENE 6 reagent (Roche Diagnostics) and Jet PEI transfection reagent (PolyPlus transfection France) and then incubated for 48 h.

**Immunoprecipitation Experiments**—Cultured cells were lysed in a buffer containing 1 mM sodium orthovanadate, 1× Complete® (Roche Applied Science), 0.5% Nonidet P-40, 0.5% cholic acid, 100 mM NaCl, 10 mM Tris, and 10 mM EDTA, pH 7.6. After a 10-min centrifugation at 1,500 rpm, cell lysates were either cold methanol-precipitated, and the resulting pellets analyzed by Western blotting after protein counting (Bio-Rad protein assay), or immunoprecipitated with different antibodies as specified under “Results.” The antigen-antibody complexes were collected by protein G-agarose beads (GE Healthcare), as specified under “Results.” The antigen-antibody complexes were analyzed by Western blotting after protein counting (Bio-Rad). Alexa Fluor® 568 and Alexa Fluor® 488 (Invitrogen) were used 1:250 for immunofluorescence. Anti-VHIs antibody conjugated with Alexa 568 (Invitrogen) was used at 1:100 for immunofluorescence. Substances and specific drugs used in this paper were from Sigma.

**Centrosome Purification**—The isolation of centrosomes from cells was as described previously (34). Cells (1–3×10^7) were treated with 5 μg/ml cytochalasin B and 10 μg/ml nocodazole for 2 h at 37°C and then harvested, centrifuged, and washed with a buffer containing 10 mM Tris, pH 7.4, 150 mM NaCl, and 8% (w/v) sucrose. Cells were then lysed in 10 ml of lysis buffer containing 1 mM Hepes, pH 7.2, 0.5% Nonidet P-40, 0.5 mM MgCl₂, 0.1% 2-mercaptoethanol, and protease inhibitors (Complete® from Roche Applied Science) and centrifuged at 2,500 × g for 10 min. The supernatant was adjusted to 10 mM Hepes, digested with 2 units/ml DNase I for 30 min on ice, then underlaid with 60% sucrose solution (in 10 mM Pipes, pH 7.2, 0.1% Triton X-100, 0.1% 2-mercaptoethanol), and centrifuged at 10,000 × g for 30 min. Centrosomes sedimented onto the cushion were resuspended and loaded onto a discontinuous gradient consisting of 70% sucrose at the bottom followed by 50 and 40% layers and centrifuged at 120,000 × g for 1 h. Fractions of 600 μl were collected in 1 ml of 10 mM Pipes buffer, pH 7.2. Centrosomes were obtained by centrifugation at 15,000 rpm for 10 min (35).

**Immunoelectron Microscopy**—H4 cells were embedded in 12% gelatin, 2.3 mM sucrose and frozen in liquid nitrogen. Ultrathin cryosections were obtained by a Reichert-Jung Ultracut E with FC4E cryoattachment and collected on copper-Formvar carbon-coated grids. Immunogold localization was performed using the antibody for GRB2 C23 from Santa Cruz Biotechnology (Santa Cruz, CA) and 10 nm protein-A-gold-conjugated. All samples are examined on a Philips CM10 or Fei Tecnai 12G2 electron microscope.

**Immunocytochemistry, FRET Studies**—Wild type H4 cells and transfected H4 cells grown to 80% confluence on slides were fixed with 4% paraformaldehyde for 15 min, blocked with 0.1 M glycine, and treated with 0.1% Triton X-100 in PBS for 3 min. After removal of the detergent, cells were then incubated with the different primary antibodies in PBS plus 3% bovine serum albumin for 1 h and finally incubated with antigen-specific secondary antibodies (Alexa 488- or 568-conjugated, from Invitrogen). Cells were then mounted by Mowiol and analyzed on an MRC 1024 ES confocal microscope (Bio-Rad), equipped with a Nikon Eclipse TE 300 inverted microscope with a ×60 objective lens. Fluorescent resonance energy transfer (FRET) was investigated through spectral analysis and then measured using the acceptor photobleaching method. FRET experiments have been performed on a Leica TCS SP2, AOBs spectral system operating with a 20-milliwatt argon laser (for 488 nm laser line) and a 1.2-milliwatt He-Ne laser (for 543 nm laser line). The FRET couples used were Alexa 488 as donor (λ_em 520 nm) and Alexa 568 as acceptor (λ_em 600 nm), the Forster radius being 62 Å, or EGFP as donor (λ_ex 499 nm, λ_em 502 nm) and Alexa 568 as acceptor (Forster radius = 60 Å). Emission spectra were collected by exciting the donor molecules at 488 nm (laser power, 10%) and acquiring the fluorescence spectrum from 500 to 700 nm with a bandwidth of 10 nm to check the presence of acceptor fluorescence because of energy transfer. To verify the occurrence of FRET, we performed acceptor photobleaching using 543 nm laser line (laser power, 100%) for 5–10 min, the variability depending from cell to cell. Comparing the donor emission spectrum before and after the complete acceptor photobleaching (acquired in the same conditions), it is possible to observe a significant change of the donor peak depending on the energy transfer. To control the bleaching, we also collected acceptor emission spectra exciting at 543 nm (laser power, 15%) and collecting the fluorescence from 550 to 750 nm, bandwidth of 10 nm, before and after acceptor photobleaching. To determine FRET efficiency, we performed acceptor photobleaching experiments simply.
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collecting donor and acceptor emission intensities measured in different cells. To acquire donor fluorescence we opened the donor channel from 500 to 540 nm (\(\Delta \lambda_{em} = 520\) nm), and for the acceptor channel we collected the fluorescence from 590 to 630 nm (\(\Delta \lambda_{em} = 603\) nm). By analyzing donor fluorescence intensity before (\(I_{DA,pre}\)) and after the complete bleaching (\(I_{DA,post}\)) of the acceptor molecules, it is possible to evaluate the efficiency (\(E\)) as shown in Equation 1,

\[
E = 1 - \frac{I_{DA,post}}{I_{DA,pre}} \quad \forall \frac{I_{DA,post}}{I_{DA,pre}} > I_{DA,pre} \quad \text{(Eq. 1)}
\]

No FRET was observed if the primary or secondary antibody was omitted. Further controls are described under “Results.”

RESULTS

APP-GRB2 Interaction in Cultured Cells—To characterize the interaction occurring between APP and GRB2, we used mouse embryonal fibroblasts not expressing endogenous APP or APP-like proteins (MEFapp). As a first approach we transfected MEFapp cells with human APP695, or its Y682A and Y687A tyrosine mutants, which may modulate the interaction with GRB2 (29), and we performed a series of immunoprecipitations (IP) and co-IP experiments. Cell lysates were immunoprecipitated with an antibody specific for the C terminus of APP (51-2700) and, after Tris-Tricine SDS-PAGE, were immunoblotted with the same antibody.

These experiments showed that APP695 is fully processed independently on the tyrosine mutation tested, as demonstrated by the presence of mature and immature APP isoforms (Fig. 1A). On the other hand, the co-IP of APP with an anti-GRB2 antibody was dependent on Tyr-682 but not on Tyr-687 (Fig. 1A). Accordingly, the mutation Y653F does not hamper the possibility to interact with GRB2, whereas the double mutant Y682A/Y687A abolished the interaction (Fig. 1A). Conversely, IP with antibodies specific for the C terminus of APP (51-2700 and F25608) co-immunoprecipitated GRB2 (Fig. 1B, top panel). As expected, this interaction appears to be dependent on Tyr-682 and not on Tyr-687 (Fig. 1B, bottom panel), thus confirming the specific interaction of APP-GRB2 and the importance of the Tyr-682 phospho-site. To further confirm the occurrence of APP-GRB2 direct interaction, we performed pulldown experiments using a Sepharose-conjugated GRB2-GST purified fusion protein. As shown in Fig. 1C, GRB2-GST fusion protein pulled down APP695 only in cell lysates from APP695-transfected MEFapp cells. To test whether the APP-GRB2 interaction also occurs between endogenous proteins, we performed IP experiments in H4 human cells. In this cell line, the APP 51-2700 antibody co-precipitated all isoforms of APP and GRB2. Conversely, the antibody to GRB2 co-precipitated both APP and GRB2 as well (Fig. 1D).

Localization of APP-GRB2 Interaction into the Cell—In order to identify the intracellular site of APP-GRB2 interaction, we performed immunofluorescence experiments and confocal microscopy analysis (36) in human H4 cells. The two antigens co-localized in a discrete, intensely fluorescent spot in the nuclear area reminiscent of the centrosomal region (Fig. 2A). GRB2 is present at the centrosome, in the cytosol, and also at the plasma membrane in any cell type we have so far tested (H4, SH-SY5Y, HEK293, C6, rat cortical astrocytes and neurons, and mouse and human fibroblasts), although with heterogeneous distribution among each different cell type. APP, detected by a specific antibody directed to the N-terminal region, was also present at the centrosome, in structures resembling the ER, in the perinuclear region, and at the cell surface (Fig. 2A). Although the peak of co-localization is at the centrosome, we observed a co-localization also in the punctate cytosolic staining, in the perinuclear region, and also partially at the plasma membrane. The proof that GRB2 localized in the centrosome area stemmed from a double immunofluorescence experiment using antibodies for \(\gamma\)-tubulin, a typical centrosomal marker (37). \(\gamma\)-Tubulin co-localized with GRB2 in a large dot next to the nuclear region (Fig. 2A). Similar data were obtained using pericentrin as a centrosomal marker. To further prove that GRB2 and APP co-localized in the centrosome area, we transfected H4 cells with vectors encoding for a histidine-tagged GRB2 construct (GRB2-his) and an EGFP-tagged APP695 (APP-EGFP). Immunostaining with an antibody specific for the histidine tag Alexa 568-conjugated showed that a fraction of both transfected proteins co-localized in the perinuclear region and at the centrosome (Fig. 2A). These experiments indicate that in human H4 cells APP and GRB2 co-localize in scattered cytosolic structures and mainly at the centrosome.

The centrosome is a very peculiar region of the cell, characterized by a pair of centrioles surrounded by a cloud of amorphous material (i.e. the pericentriolar material) whose origin and composition are unclear. Previous reports have identified signaling proteins and membrane proteins in this region (for a review see Ref. 30). To better define the localization of GRB2 at the centrosome, we carried out immunogold labeling and electron microscopy studies using a specific antibody for GRB2 (C23). These experiments showed that GRB2 antibody labeled the outer periphery of centrioles, the pericentriolar electron-dense material, membrane-bound vesicles, and tubulo-vesicular structures (Fig. 2B, top panels). Double immunogold labeling with antibodies to \(\gamma\)-tubulin and GRB2 on cross-sections of centrioles in the centrosome area confirmed these results (Fig. 2B, bottom panels).
Characterization by Fluorescence Resonance Energy Transfer (FRET) of APP-GRB2 Interaction at the Centrosome—The co-localization studies suggested that the GRB2-APP interaction may occur in the centrosome area. We sought for more direct evidence of this site-specific interaction by the way of FRET experiments combining spectral analysis with acceptor photobleaching (38). FRET allows measuring the vicinity between two molecules within 10 nm, because of the radiationless energy transfer between the donor and the acceptor. We used antibodies for APP (N terminal (Sigma), C20, and 13-0200), coupled to a secondary antibody conjugated to Alexa 488 as donor, and antibodies for GRB2 (either C23 or E1) coupled to a specific secondary antibody Alexa 568 conjugated as acceptor molecule.

Spectral analysis of immunolabeled centrosomes showed that excitation of the donor (APP) at 488 nm allowed the appearance of a first emission peak above 500 nm (i.e. the normal emission peak for Alexa 488), followed by a second peak above 600 nm that represents the emission from GRB2-Alexa 568 upon energy transfer from the donor molecules, suggesting the occurrence of FRET (Fig. 3A, black line). To ascertain the incidence and the amount of FRET, we then performed acceptor photobleaching experiments in the same conditions (39). After photobleaching of the acceptor (GRB2-Alexa 568), and a second scan at 488 nm, we observed an increase of the donor peak above 500 nm (Fig. 3A, red line). These results implied that APP-Alexa 488 molecules can no longer transfer their photons to the photobleached acceptor, suggesting that the two molecules were in close proximity at the centrosome. Statistical analysis and measurement of FRET by steady state acceptor photobleaching experiments in centrosomes from different cells showed that the APP-GRB2 interaction at the centrosome has an efficiency of 25 ± 4% (n = 20 cells on at least three different experiments).

Controls for FRET experiments included the following: (a) to verify the complete acceptor photobleaching, we collected the emission spectra directly exciting the acceptor molecules (543 nm) before and after the bleaching (Fig. 3, right panels); (b) to exclude phenomena other than FRET, we collected the intensity in a region centered in another centrosome in close proximity (either in the same cell or in a second cell nearby), where we did not bleach the acceptor (Fig. 3B); (c) we checked the fluorescence intensity inside the bleached region but outside the centrosome (Fig. 3C); (d) we measured the fluorescence intensity in a region outside the bleaching region within the cell (Fig. 3D); (e) we controlled background regions (background is reported for each graph). FRET was observed in none of these conditions. Altogether these data indicate that APP and GRB2 co-localize and interact with each other in the centrosome of H4 cells.

Fig. 4A shows an example of FRET reflecting the interaction between APP and GRB2 in the centrosome. H4 cells were immunolabeled as described above and analyzed before and after photobleaching by confocal analysis. Upon photobleaching, a significant enhancement of donor (APP-Alexa 488) emission intensity, with a parallel decrease of acceptor (GRB2-Alexa 568) emission intensity, indicated resonance and interaction between APP and GRB2 in the centrosome. Analogous experiments using the FRET couple APP-EGFP and GRB2-his conjugated to Alexa 568 gave similar results (data not shown).

Under the same conditions, we performed also experiments using γ-tubulin-Alexa 488 as donors and APP-Alexa 568 or GRB2-Alexa 568 as acceptors. We observed that the centrosomal marker γ-tubulin co-localized with both APP and GRB2,
confirmed the localization of PS1 in centrosomes, as well as in Golgi-like structures, and at the cell surface. As expected, both the polyclonal C23 and the monoclonal E1 antibodies to GRB2 co-localized with the N terminus (Fig. 5A) and the C terminus (Fig. 5B) of PS1, at the centrosome. In addition, the co-localization with γ-tubulin further confirmed the PS1 expression in the centrosome (Fig. 5C). To test whether the PS1-GRB2 co-localization in the centrosome was associated with a direct interaction between the two proteins, we transfected MEF cells PS1 and PS2 null (MEFps−/−) with vectors encoding for either PS1 wild type or with PS1 carrying familial mutations of Alzheimer disease (i.e. H163R and L286V), tagged with a C-terminal V5 tag. Cell lysates were analyzed by a combination of IP and immunoblotting. These experiments showed that the antibody for GRB2 was able to precipitate the V5-tagged C-terminal PS1 fragment migrating at 20 kDa (Fig. 5D). Similarly, the antibody to the PS1-C-terminal V5 tag co-precipitated GRB2 protein migrating at 25 kDa (Fig. 5D). The expression of mutated PS1 proteins carrying familial mutations did not hamper the interaction with GRB2 (Fig. 5D). In parallel experiments we verified by crossed IP and immunoblotting the reciprocal interaction between endogenous PS1 and GRB2 in H4 cells. The IP with an antibody for PS1 co-precipitated GRB2 at 25 kDa and similarly the IP with GRB2 co-precipitated PS1 C-terminal fragment migrating at 19 kDa (Fig. 5E). A parallel IP performed with an unrelated antibody (CD26) was done as a control for the specificity of the interaction. The relative amount of PS1 co-precipitated by GRB2 corresponds to the 1.24 ± 0.33% of the total PS1 content, whereas the relative amount of GRB2 co-precipitated by the antibody for PS1 (N terminus) is 1.8 ± 0.2%. However, we must consider that under our conditions, the efficiency of GRB2 IP is around 7.8% and for PS1 IP is 6.5%. Altogether, these experiments confirm the presence of PS1 in the centrosomal area and suggest a relevant interaction with GRB2.

As shown previously for APP, to prove the centrosomal interaction PS1-GRB2, we performed FRET experiments as described above (Fig. 3). H4 cells were immunolabeled with an antibody for GRB2 coupled to a secondary antibody Alexa 568 conjugated as acceptor, and the two antibodies for the C terminus of PS1 (C20) or for PS1 N terminus both conjugated to antibody for GRB2 coupled to a secondary antibody Alexa 488 and used alternatively as donors. Spectral analysis showed a significant increase of the donor peak after a complete acceptor photobleaching at the centrosome (Fig. 6). Similarly, the antibody for PS1 co-precipitated GRB2 with an efficiency of 23 ± 5% (n = 25 cells) at the centrosome, whereas the couple PS1 N-terminal GRB2 showed an efficiency of 18 ± 6% (n = 13 cells). The same experiments were done also with the couples PS1 C-terminal Alexa 488 or PS1 N-terminal Alexa 488 as donors, and γ-tubulin-Alexa 568 as acceptor. Besides their co-localization, after photobleaching of γ-tubulin, the intensity of the signal from PS1C or N-terminal labeling did not change (Fig. 6, B and D), confirming the specificity of the interaction PS1-GRB2. C-terminal PS1 and γ-tubulin (Fig. 6B) showed a nonsignificant increase of the donor peak (E = 4 ± 4%) (n = 18 cells). Therefore, PS1-GRB2 directly and specifically interacts in the centrosome.

To obtain direct biochemical proof of the presence of APP, PS1, and GRB2 in the centrosome, we performed Western blotting
analysis on purified centrosomes. Centrosome-enriched fractions were prepared from H4 cells after treatment with nocodazole and cytochalasin B to depolymerize the microtubule-microfilament network. Cells were lysed, and the post-nuclear supernatant was subjected to sucrose density gradient centrifugation procedure as described previously (34) with minor modifications. The fractions were collected and analyzed by immunoblotting with different antibodies. In this protocol, the centrosomes were enriched in the 50–70% sucrose region of the discontinuous gradient corresponding to fractions 7–9. As shown in Fig. 7, H9253-tubulin was primarily recovered in these fractions, although some H9253-tubulin also sedimented in the lower sucrose fractions corresponding to the cytoplasmic pool of the protein (34). APP, PS1, and GRB2 also co-sedimented with the centrosome fractions 7–9, whereas Golgin-97 immunoreactivity was detected only in whole cell lysates (Fig. 7, + lane) and was absent in centrosomal fractions, indicating the absence of Golgi vesicles from our samples, which represent the most common form of contamination in these preparations.

APP Regulates Phospho-ERK1,2 Levels—Because GRB2 is mainly involved in the regulation of growth factor signaling targeted to ERK1,2 activation, we investigated whether the centrosomal interactions with APP and/or PS1 would influence ERK1,2 phosphorylation. We initially verified whether changes in the levels of APP in vitro would affect the phosphorylation of ERK1,2. We increased APP levels by either transfecting H4 cells with vectors encoding for APP695 or by silencing the mRNA of H9252-secretase BACE1. On the contrary, we decreased APP content overexpressing BACE1 to increase APP cleavage. RT–PCR revealed that both H4 and H4-BACE expressed BACE1 mRNA (Fig. 8 A). In particular, the expression of the engineered BACE1 protein was verified by direct immunostaining with an anti-FLAG antibody and by an anti-BACE1 C-terminal antibody upon Western blotting (Fig. 8 A). These data show that in H4 cells expressing BACE1 (H4-BACE), there is effectively a higher amount of BACE1 protein as expected.

To provide further evidence of the enhanced BACE1 expression and activity on APP processing, we monitored the amount of CTFs upon BACE1 overexpression as described previously (42). Cleavage of APP by BACE1 generates C99 and C89, whereas cleavage by α-secretase results in C83, all of which can be resolved by Tris-Tricine SDS-PAGE, along with their phosphorylated isoforms (43) (Fig. 8B).
In our conditions, the analysis with an antibody specific for the very N terminus of C99 (44, 45) confirms that the first two bands represent C99 and its phosphorylated isoform (43). The increased expression of BACE1 resulted in a significantly enhanced CTFs formation, with cleavages at position 1 and 11 predominating (Fig. 8B), as demonstrated by the increased amount of phospho-C99, C99, and C89 fragments in H4-BACE cells, compared with H4 wild type cells.

We then determined the basal level of expression of phospho-ERK1,2 (pERK1,2) upon overexpression of APP and/or BACE1. H4 cells transfected with vectors encoding for APP695 showed a synchronous up-regulation of both APP and pERK1,2 after 16 h of starvation in medium without serum (Fig. 8C). The co-transfection with BACE1 and its overexpression decreased significantly both APP and pERK1,2 levels (Fig. 8C). The densitometric quantification in different experiments showed a significant enhancement of pERK1,2 levels upon overexpression of APP695. When instead BACE1 was overexpressed along with APP, we measured a significant reduction of pERK1,2 activation (Fig. 8D). The overall effect resulted in a significant up-regulation of cell growth, measured by [3H]thymidine incorporation, in cells overexpressing APP695, which is negatively modulated by BACE1 (Fig. 8E). To verify the specificity of this signaling, we measured the levels of pERK1,2 in cells transfected with the APP mutant Y682A, which is processed and cleaved to form CTFs as the wild type and the Y687A mutant (Fig. 9A), but does not interact with GRB2 (Fig. 1A and B). In these experiments we observed that the expression of the APP695 wild type or the Y687A mutant enhanced pERK1,2, whereas the tyrosine mutation at residues 682 significantly decreased the phosphorylation of ERK1,2 (Fig. 9B). These experiments ruled out also the possibility that the positive effect on pERK1,2 exerted by APP695 would be generated following an enhanced formation of soluble APP N-terminal fragments, by a sort of paracrine effect. In fact, the APP mutant Y682A, which is normally processed and cleaved to form CTFs as the wild type isoform, does not activate ERK1,2, thus suggesting that the mechanism, rather than a proliferative effect of soluble APP, is linked to the C-terminal region of APP and it is GRB2-related. We then verified the effect of the overexpression of BACE1 compared with its silencing by RNA interference. The overexpression of BACE1 diminished pERK1,2, and its silencing with a specific siRNA (32) significantly up-regulated ERK1,2 phosphorylation (Fig. 9C), suggesting a direct correlation between APP amount, BACE1 activity, and activation of pERK1,2. The treatment with PD98059, a specific inhibitor of MEK kinase, was used as control for the phosphorylation of ERK1,2 (Fig. 9D). Altogether these data suggest that APP, through its interaction with GRB2, enhances ERK1,2 phosphorylation in a manner that in part depends on its cleavage by BACE1.
APP and PS1 Modulate ERK1,2

FIGURE 8. APP modulates ERK1,2 activation. A, H4 cells were engineered to overexpress BACE1 as shown by RT-PCR for BACE1 and BACE2, by Western blotting for BACE1, and for its C-terminal FLAG; B, analysis of CTFs by direct Western blotting after being IP with the antibody 51-2700 for the C terminus of APP or with the antibody R3659 for the N terminus of C99. Cells in which BACE1 is overexpressed either transiently (tr) or in a stable (st) manner show an increment of phospho-C99 (phC99), C99, and C89, in comparison with the wild type (wt) cells. C83 levels are unaffected. C, upon overexpression of APP695, both APP and pERK1,2 levels are up-regulated, whereas both are down-regulated when a BACE1 expressing vector is co-transfected. D, almost 2-fold increment in phospho-ERK1,2 levels is measured by densitometric analysis in six different experiments upon APP695 transfection. This increment is instead significantly down-regulated if BACE1 is co-transfected. E, [3H]thymidine incorporation assay shows that DNA synthesis is significantly enhanced in H4 cells upon APP695 overexpression, although it is reduced if BACE1 levels are simultaneously enhanced.

FIGURE 9. ERK1,2 up-regulation depends on APP. A, mutants Y682A and Y687A of APP695 are processed and cleaved as the wild type (wt) isoform upon transfection in APP/APLPs null MEF cells. B, expression of APP695 wild type (wt) or Y687A mutant results in a parallel up-regulation of phospho-ERK1,2. The expression of the Y682A mutant instead results in a significantly reduced activation of pERK1,2. C, phosphorylation of ERK1,2 is enhanced by the overexpression of APP695 and by the RNA silencing of the constitutive BACE1 and is down-regulated by the overexpression of BACE1 and by the treatment with the MEK kinase inhibitor PD98059 (PD). ERK1,2 and tubulin are shown as loading controls, whereas the transfection with an unrelated vector (vec) is shown as control for the specificity.

DISCUSSION

Our data show that APP and PS1 modulate the centrosomal mitotic activity of ERK1,2, through their interaction with GRB2. Although APP and PS1 are pivotal proteins in AD generation, at present it is unclear whether this signaling has a relevance for AD, or if it is rather involved in the normal cell cycle regulation.

To date the most relevant role for APP or PSs on AD development is linked to the “amyloid hypothesis,” which considers the formation of Aβ peptides the central event of the neurodegenerative (2, 6). In this view, PSs are considered essentially as γ-secretase regulatory molecules, APP the substrate of the γ-secretase action, and corollary or secondary events are Tau-linked pathology and gliosis. The theory foresees that familial mutations in both proteins enhance and accelerate Aβ formation, triggering the pathogenic cascade that leads to neurodegeneration (47). A second theory, complementary to the amyloid hypothesis, proposes that APP and PSs may modulate a yet unclear cell signal, the disruption of which may induce cell cycle abnormalities, neuronal death, eventually Aβ formation, and finally dementia (14). In support of this theory are the following: (a) cell cycle defects represent a major

Presence and Localization of Phospho-ERK1,2 at the Centrosome in Mitotic Cells in a PS1- and APP-dependent Manner—

Our data suggest that a target for the signaling of APP and PS1 is the kinase ERK1,2, which is known to be down-regu-
neuropathological feature in AD and in transgenic animal models of AD (48–51); (b) APP and PSs have been associated with chromosome missegregation, trisomy 21 mosaicism (41, 52); and (c) both proteins interact with a complex protein network with a clear relevance for signal transduction mechanisms (13, 19, 53, 54). In this context, the C-terminal domain of APP has a central role because of the presence of the \(^{682}\)YENPTY\(^{687}\) motif that represents the docking site for multiple interacting proteins involved in cell signaling (22, 29, 31, 55–64). Among different APP-interacting proteins, only ShcA and GRB2 act through a specific phosphorylation step at Tyr-682 (29), the intimate regulation of which is still under investigation. GRB2 and ShcA, which usually connect growth factor receptors to mitogen-activated protein kinase (MAPK) signaling, may act independently from each other, and APP has the capability to recruit GRB2 directly or indirectly through ShcA (21, 29, 65).

Our data show that APP regulates pERK1,2 levels, its phosphorylation/translocation to the centrosome, and the cell proliferation rate. We demonstrated that the proteolysis of APP regulates ERK1,2 activation as well, because BACE1 overexpression enhances APP cleavage and CTFs formation and reduces ERK1,2 phosphorylation in H4 cells. Noteworthy, APP-GRB2 interaction is present in proliferating cells, and it is abolished in apoptotic conditions, where the interaction C99-GRB2 instead prevails (65). Considering that both complexes might influence pERK1,2 activity, it would be interesting to observe if selective \(\gamma\)-secretase inhibitors may modulate pERK1,2 activity in apoptotic models. Altogether these data suggest a dual and opposite pERK1,2 function as observed previously in other systems, where enhanced pERK1,2 levels can either enhance proliferation or cause growth arrest modulating different cyclin kinases (66). As experienced in other tyrosine kinase receptor-dependent signaling, a selective phosphorylation at Tyr-682 of APP regulates both the interaction with GRB2 and ERK1,2 phosphorylation, suggesting a typical receptor/transducer-like activity for APP aimed at the regulation of ERK1,2 through its interaction with GRB2.

In this scenario, the signaling activity of APP is complicated by the fact that PS1 as well interacts with GRB2 and modulates ERK1,2 signaling as shown here and in previous works (16, 17). Thus, the hypothesis is that both APP and PS1 participate on the same signaling pathway through GRB2, which become the first identified signaling protein that interacts with both gene products responsible for the generation of familial AD. Although the presence of PSs in the centrosome has been reported previously (40, 41), the simultaneous presence of PS1, APP, and GRB2, and most importantly their reciprocal interaction, represents a new finding that might open new perspectives for the investigation of PSs and APP function, as well as for the understanding of GRB2 role in cell signaling and cell cycle regulation. In fact, the centrosome is a crucial region for microtubule nucleation, cell cycle progression, migration, cytokinesis, and cellularization (30).

The centrosome is a central component of the cell cycle machinery of eukaryotic cells, and centrosome amplifications or perturbations of centrosomal proteins are linked to cell cycle misregulation and cancer (67, 68). The microtubule cytoskeleton provides a highly dynamic intracellular framework upon which trafficking of vesicles, individual proteins, and macromolecular complexes can occur. Estimates suggest that the centrosome includes hundreds of proteins involved in cell cycle regulation, protein degradation, transporters, kinases and phosphatases, microtubule-associated proteins, signaling proteins, membrane receptor signaling and scaffold proteins that serve as docking sites for a growing number of regulatory activities (30). Our immuno-EM study (Fig. 2B) shows the presence of 50–70 nm vesicles in the pericentriolar material, whose origin is still unclear, although the presence of late endosome recycling vesicles, Rab11, and caveolin-positive vesicles has been described previously in the pericentriolar compartment (69, 70). These vesicles may be carriers of proteins and receptors stemming from the cell surface or from other cell compartments.

The centrosomes are also an important location for proteolytic degradation of proteins known as “aggresomes,” which are proteinaceous inclusions formed at the centrosome in response to proteolytic stress (68, 71). At present our observations...
exclude that the complex between PS1, APP, and GRB2 represents a degradative pathway localized at the centrosome. Our evidence shows that APP, PS1, and GRB2 are not ubiquitinated and are detectable in the centrosome at different stages of the mitotic cycle in cells that do not overexpress proteins, independently on treatment with proteasome inhibitors and in a manner that instead depends on microtubule stability (data not shown).

Because many regulatory molecules are found at centrosomes, it has been proposed that centrosomes serve as signaling machines that modulate different cell functions (30). In particular, considering that GRB2, APP, PS1, and pERK1,2 are all detectable in mitotic centrosomes, it is conceivable that these structures might anchor signal transduction pathways, integrate signals, and facilitate the conversion of these signals into cellular functions. Our data suggest that PSs and APP may take part in these signaling complexes, whose function is related to the activation of ERK1,2 and to modulate the cell cycle.

In summary this study represents an initial step toward the comprehension of a novel signaling mechanism, in which pivotal proteins for AD genesis are involved. It is tempting to speculate that ERK1,2 activation, mediated by both APP and PS1, may be relevant for Tau phosphorylation and neurofibrillary tangles formation, considering that centrosomes are essential for microtubule stability and assembly. Nonetheless, the hypothesis that pERK1,2 is involved in the activation of a parallel proapoptotic pathway, possibly through an enhanced Aβ formation, must be considered as well. Further analyses are needed to identify the effect of APP or PSs FAD mutants on the centrosomal phenotype, to verify whether PS1 and APP compete each other for the GRB2-linked signal, and to understand the main consequences of their centrosomal signaling activity in the human brain. Among the different centrosomal functions, it will be of importance to also verify the effect on glial proliferation and cell cycle regulation in postmitotic neurons where aberrant cell cycle re-entry may occur (14). It also remains to be elucidated whether the centrosomal pool of APP and PS1, together with GRB2, is involved in intracellular amyloid-β formation as well, and whether other components of the γ-secretase complex are implicated in the interaction, because in our conditions APP and PS1 are in close proximity also at the centrosome. However, we must bear in mind that different APP or PS1 interactors are able to modulate the formation of Aβ (72–75), and therefore that parallel signals, often connected each other, may modulate this event that is difficult to study in other cell compartments, where APP and PS1 may also co-localize and interact. This hypothesis is also suggested by the significant amount of proteins engaged in co-IP experiments. A deeper comprehension of these aspects of APP and PS1 life is necessary and would open new possibilities for a therapeutic intervention for AD. In this respect it is of particular importance that the Tyr-682 phosphorylation site regulates GRB2 interaction and ERK1,2 activation. The investigation on kinases and phosphatases that activate this site would represent a putative spot of intervention to facilitate or block the underlying signal.

The last aspect, which is unrelated to AD development, regards the central role of GRB2 in cancer. The etiology and progression of a variety of human malignancies are linked to the deregulation of receptor tyrosine kinases that may recruit either ShcA or GRB2 adaptor proteins to activate their signaling. In this study we show that GRB2, besides its well known cytosolic localization, is also recruited at the centrosome of the cell. Considering the pivotal role of GRB2 in oncogenic proliferation and metastasis, and considering the complexity of the cross-talk between receptors in tumorigenesis, we cannot exclude that APP and PSs might influence GRB2 activity or localization and modulate the mitogenic or metastatic potential of tumor cells, especially in view of the potential involvement of PS1 in oncogenic pathways, which is suggested by the fact that receptors and transducers cleaved by γ-secretase are involved in oncogenic signaling such as Notch (77, 78), ErbB-4 (10, 79), Wnt (12, 80, 81), and CD44 (9).

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