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Integrins Mediate β-Amyloid-Induced Cell-Cycle Activation and Neuronal Death

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Early intracellular events responsible for cell-cycle induction by β-amyloid (Aβ) in neurons have not been identified yet. Extracellular signal–regulated kinases 1/2 (ERK1/2) have been identified in this pathway, and inhibition of ERK activity prevents cell-cycle activation and reduces neuronal death induced by Aβ. To identify upstream events responsible for ERK activation, attention has been focused on integrins. Treatment of SH-SY5Y cells, differentiated by long-term exposure to 10 μM retinoic acid with a neutralizing anti-α1-integrin antibody significantly reduced Aβ-induced neuronal death. However, cell-cycle analysis showed that treatment with anti-α1-integrin per se produced changes in the distribution of cell populations, thus hampering any effect on Aβ-induced cell-cycle activation. 4-Amino-5-(4-chlorophenyl)-7(t-butyl)pyrazol(3,4-D)pyramide, an inhibitor of src protein kinases that colocalizes with focal adhesion kinase (FAK) and is involved in integrin signaling, was effective in reducing activation of the cell cycle and preventing induction of neuronal death by Aβ while inhibiting ERK1/2 phosphorylation. Similar results were obtained when FAK expression was down-regulated by siRNA silencing. The present study identifies a sequence of early events in the toxic effect of Aβ in neuronal cultures that involves interaction with integrins, activation of FAK/src, enhanced phosphorylation of ERK1/2, and induction of the cell cycle, all leading to neuronal death.

Key words: cell cycle; neuronal death; FAK; ERK

Although neurons have been classically recognized as postmitotic cells, evidence has accumulated in recent years that injured neuronal cells have the ability to activate the cell cycle. Such a phenomenon also has been described as occurring in the brains of patients with Alzheimer's disease (AD), strongly supporting a role for cell-cycle abnormalities in the pathogenesis of AD. Thus, reexpression of several cell-cycle-related proteins, such as cdc2, cyclin B1, cdk4, cyclin D, and p16, has been reported in sick as well as in vulnerable neurons in the brains of AD patients (Nagy et al., 1997; Vincent et al., 1997; Busser et al., 1998; McShea et al., 1999; Husseman et al., 2000). Similar reexpression of cell-cycle-related proteins has been also reported in experimental mouse models of AD (Herrup et al., 2004).

A focused analysis of the reappearance of cell-cycle markers and their relationship to cell-cycle activation revealed that primary cultures of rat cortical neurons challenged with $A\beta_{(25–35)}$ enter the cell cycle, as demonstrated by the appearance of a cell population in the S phase of the cycle. This phenomenon is accompanied by sequential and ordered expression of cell-cycle proteins such as phospho-Rb and cyclins and is thought to lead to neuronal apoptosis (Copani et al., 1999).

Although the intracellular pathway of cell-cycle activation and neuronal death by Aβ has been drawn, early transducing mechanisms operating in the induction of the cell cycle by Aβ have not been clearly identified and described.

To better understand the early mechanisms underlying Aβ-induced cell-cycle activation, we used a human neuroblastoma cell line, SH-SY5Y cells, differentiated by a 5-day exposure to retinoic acid. We previously showed that in this cell line (1) $A\beta_{(25–35)}$ induces modifications in the distribution of cells in different phases of the cell cycle, an event that precedes neuronal apoptosis; and (2) the extracellular signal–regulated kinase (ERK) pathway is involved in this effect (Frasca et al., 2004). To analyze possible pathways upstream of ERK, we focused our attention on integrins that, among others, have been identified as potential mediators of the early response to Aβ (Kowall et al., 1991; Boland et al., 1995; Sabo et al., 1995; Yan et al., 1996; Yaar et al., 1997). Binding to integrins resulted in the consecutive activation of tyro-

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sine kinases and mitogen-activated protein kinase (MAPK). In fact, stimulation of integrins induced autophosphorylation and activation of focal adhesion kinase (FAK), which through the MAPK- and phosphatidylinositol-3 (PI3) kinase pathways, links integrin signaling to activation of ERK1/2 and JNK (Arendt, 2003). We now report that integrins and the ensuing transducing pathways are in fact involved in Aβ-induced cell-cycle induction, thus representing a potential pharmacological target against Aβ toxicity.

MATERIALS AND METHODS

Cell Culture and Reagents

SH-SY5Y human neuroblastoma cells were grown in DMEM containing 10% FCS and antibiotics (all from Invitrogen, Milan, Italy). Only cells between passages 24 and 38 were used. Cells were shown to maintain their morphological features and to respond to retinoic acid differentiation and $A\beta_{(25–35)}$ toxicity in a consistent way. Cultures were differentiated for 5 days with 10 μM retinoic acid prior to serum deprivation and treatment with the Aβ analogue $A\beta_{(25–35)}$ (Bachem, Feinchemikalien AG Bubendorf, Switzerland).

$A\beta_{(25–35)}$ was solubilized in sterile, doubly distilled water at an initial concentration of 2.5 mM, stored frozen at −20°C, and used at a final concentration of 25 μM. Mouse antihuman α1-integrin domain monoclonal antibody (MAB1973, Chemicon International, Temecula, CA) was used at a final concentration of 2.5 μg/mL. 4-Amino-5-(4-chlorophenyl)-7(t-butyl)-pyrazol(3,4-D)pyramide (PP2; Calbiochem, La Jolla, CA) was used at a final concentration of 1 μM.

MTT Assay

Following $A\beta_{(25–35)}$ treatment, SH-SY5Y cells were incubated with the MTT dye solution (0.9 mg/mL final concentration) for 2 hr at 37°C. After solubilization, the formazan produced was evaluated in a plate reader (absorbance = 560 nm).

Analysis of Cell Cycle

Cell-cycle distribution was evaluated by cytofluorometric analysis. SH-SY5Y cells were detached and fixed in 70% ethanol at −20°C overnight, thus allowing fixation and permeabilization. Cells were then repeatedly washed, incubated with 100 μg/mL RNase for 1 hr at 37°C, and stained with 50 μg/mL propidium iodide for 30 min. Analysis was carried out in a Coulter Elite flow cytometer. Cell debris was gated out based on light-scatter evaluation, and analysis was restricted to cells with diploid or, when present, hypodiploid DNA. Between 10,000 and 20,000 events per sample were analyzed. For cell-cycle distribution, data were analyzed with Multicycle AV software (Phoenix Flow System, San Diego, CA).

Bisbenzimide Staining

Apoptosis was evaluated by nuclear staining with bisbenzimide, using nuclear morphology as the criterion for identifying cell death. Cells were fixed in 4% paraformaldehyde, washed in PBS, and permeabilized with Triton 0.1% for 5 min. After washing in PBS, cells were incubated with bisbenzimide (0.8 μg/mL; Sigma Aldrich, St. Louis, MO) for 30 min at 37°C, washed with H_2O, and analyzed with an Axiovert 40 fluorescent microscope (Zeiss, Arese, Italy) with a UV filter, using a 20× objective lens. Six fields per plate and 3 plates per group were examined.

The percentage of apoptotic cells was calculated on a blinded basis as the ratio of cells with fragmented or condensed nuclei/total number of cells with intact nuclei.

FAK RNA Interference

SH-SY5Y cells were differentiated for 5 days with 10 μM retinoic acid and transfected with either FAK siRNA or negative control siRNA at a final concentration of 100 nM with Lipofectamine 2000 (Invitrogen, Milan, Italy) for 5 hr according to the manufacturer's protocol. The specific siRNA sequence used for FAK was: 5′-GAAGUUGGGUUGUCUAGAAUU-3′ (MWG Biotech, Ebersberg, Germany). Validation of FAK silencing with this specific sequence was reported previously (Beierle et al., 2007) and was carried out in the present study by Western blot analysis 24 and 48 hr after transfection. The nonspecific siRNA control sequence was: 5′-AGGUAGUGUAAUCGCCUUGUU-3′ (MWG Biotech). Twenty-four hours after transfection, cells were exposed to 25 μM $A\beta_{(25–35)}$ for 30 min for assessment of ERK phosphorylation or for 24 hr for cell-cycle analysis.

Western Blotting

SH-SY5Y cells were harvested at 4°C with a lysis buffer containing antiproteases and antiphosphatases. After sonication, samples were diluted in sample buffer and boiled for 10 min. Protein separation was performed by SDS-polyacrylamide gel electrophoresis (30 mA/hr) using 60 μg of protein per lane. Proteins were transferred onto a nitrocellulose membrane (Hybond ECL, Amersham Italy) for 2 hr at room temperature using a transblot semidry transfer cell. After blocking with 3% nonfat dry milk, the membrane was incubated overnight at 4°C with rabbit polyclonal anti-phospho-p44/42 MAPK antibody (1:1,000; Cell Signaling Technology Inc., Danvers, MA) or rabbit polyclonal anti-FAK antibody (1:400; Santa Cruz Biotech. Inc., Santa Cruz, CA), then repeatedly washed and exposed to horseradish peroxidase–conjugated antirabbit IgG (1:5,000) for 1 hr at room temperature. Bands were visualized using the Supersignal West Pico chemiluminescent substrate (Pierce, Rockford, IL). To control for equal protein loading, membranes were incubated with rabbit polyclonal anti-p44/42 MAPK antibody (1:1,000; Cell Signaling Technology Inc., Danvers, MA) or mouse anti-α-tubulin (1:5,000; Sigma Aldrich, St. Louis, MO).

Statistical Analysis

All data were analyzed by one-way analysis of variance, followed by the Newman-Keuls t test for significance. $P < 0.05$ was the criterion for statistical significance.

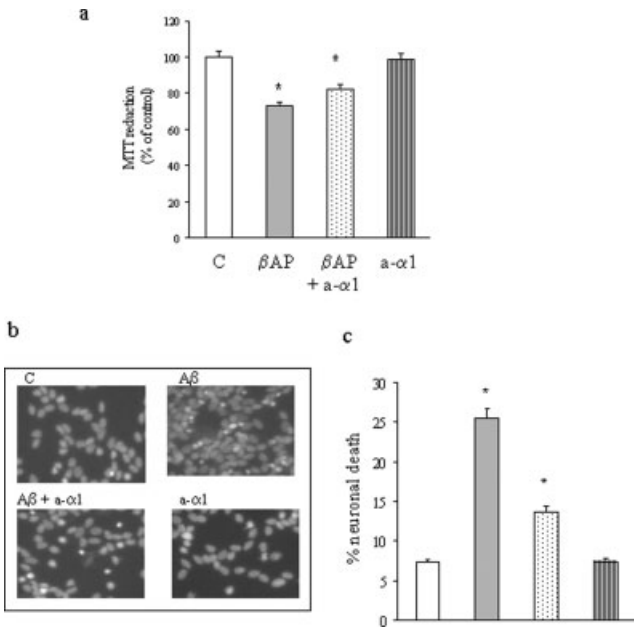

Fig. 1. Effects of α1-integrin blocking antibody on cell death in differentiated SH-SY5Y neuroblastoma cells. α1-Integrin blocking antibody (2.5 μg/mL) was added to the cultures 1 hr prior to exposure to 25 μM $A\beta_{(25–35)}$ for 48 hr. **a:** Cell viability analyzed by MTT assay. **b:** Apoptotic cell death evaluated by bisbenzimide staining. **c:** Quantitative analysis of apoptotic nuclei. Each experiment was run in triplicate, and 6 fields per plate were analyzed. Data are means ± SEMs of 3 independent experiments (⋆$P < 0.05$ vs. all other groups).

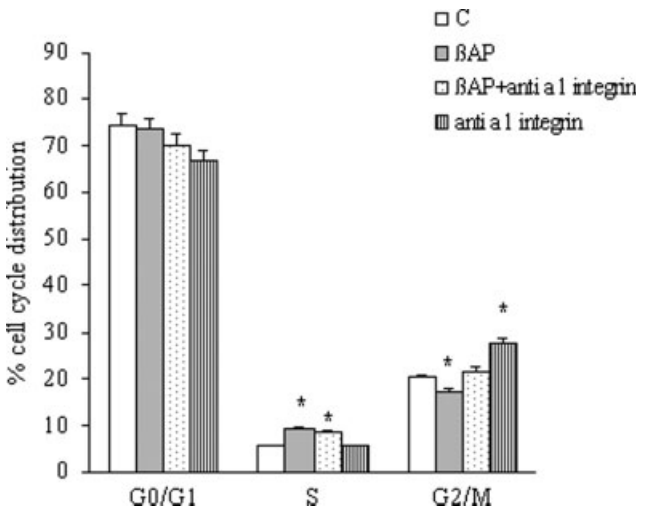

Fig. 2. Effects of α1-integrin blocking antibody on cell-cycle distribution in differentiated SH-SY5Y neuroblastoma cells. α1-Integrin blocking antibody (2.5 μg/mL) was added to the cultures 1 hr prior to exposure to 25 μM $A\beta_{(25–35)}$ for 24 hr. Cell-cycle distribution profiles were analyzed by flow cytometry following propidium iodide staining. Data are means ± SEMs of 3 independent experiments (⋆$P < 0.05$ vs. respective control).

RESULTS

To examine the involvement of integrins in Aβ-induced cell-cycle activation and neuronal death, differentiated SH-SY5Y cells were treated for 1 hr with α1-integrin blocking antibody (2.5 μg/mL) and then exposed to 25 μM $A\beta_{(25–35)}$ for 24–72 hr.

Inhibition of the α1-integrin subunit was able to reduce Aβ-induced neuronal death, as detected by the MTT assay after 48 hr of exposure to 25 μM $A\beta_{(25–35)}$ (Fig. 1a). A similar effect was observed after 72 hr of incubation (not shown). In addition, staining of apoptotic nuclei with bisbenzimide revealed about a 30% reduction in apoptosis induced by treatment with Aβ for 48 hr (Fig. 1b,c). Pretreatment of SH-SY5Y cells with 2.5 μg/mL α1-integrin blocking antibody for 1 hr prior to exposure to $A\beta_{(25–35)}$ for 24 hr did not modify the accumulation of cells in S phase induced by $A\beta_{(25–35)}$ (Fig. 2). However, the blockade of α1-integrin per se, despite no change in the viability of SH-SY5Y cells, induced a different profile of distribution of the cells in the cell cycle with an increase in the population of cells in the G2/M phase (Fig. 2).

In an attempt to demonstrate a link between the Aβ-integrin complex on the neuronal membrane and ERK1/2 activation, attention has been focused on focal adhesion kinase (FAK), which is directly activated by integrin receptors. Differentiated SH-SY5Y cells were then exposed to 1 μM PP2, a Src family tyrosine kinase inhibitor, for 20 min prior to treatment with 25 μM $A\beta_{(25–35)}$ for 30 min. Pretreatment with PP2 was able to reduce ERK1/2 phosphorylation induced by $A\beta_{(25–35)}$, as shown by Western blot analysis (Fig. 3a).

In addition, PP2 prevented the reduced cell viability induced by exposure to 25 μM $A\beta_{(25–35)}$ for 48 hr, as revealed by the MTT assay (Fig. 3b). More interestingly, under these conditions, the pattern of distribution within the cell cycle was modified. Treatment with 1 μM PP2, which per se did not modify cell populations in different phases of the cycle, abolished the accumulation in the S phase induced by $A\beta_{(25–35)}$ (Fig. 4).

To further confirm the contribution of FAK signaling to the effects of $A\beta_{(25–35)}$, SH-SY5Y cultures were treated with FAK siRNA, which significantly decreased FAK protein levels as shown by Western blot analysis. In contrast, treatment with control nonspecific siRNA did not produce any reduction in FAK expression (Fig. 5a). A similar degree of silencing was observed at either 24 hr (Fig. 5a) and 48 hr (not shown) after transfection. Hence, 24 hr after treatment with FAK siRNA, SH-SY5Y cells were exposed to $A\beta_{(25–35)}$ for 30 min to analyze activation of ERK1/2. Western blot analysis indicated that under these conditions, ERK1/2 phosphorylation induced by $A\beta_{(25–35)}$ was prevented (Fig. 5b). In parallel, 24 hr after FAK silencing, cells were exposed to 25 μM $A\beta_{(25–35)}$ for an additional 24 hr. Analysis of the cell cycle following this treatment revealed that the accumulation of SH-SY5Y cells in the S phase induced by $A\beta_{(25–35)}$ was abolished (Fig. 5c).

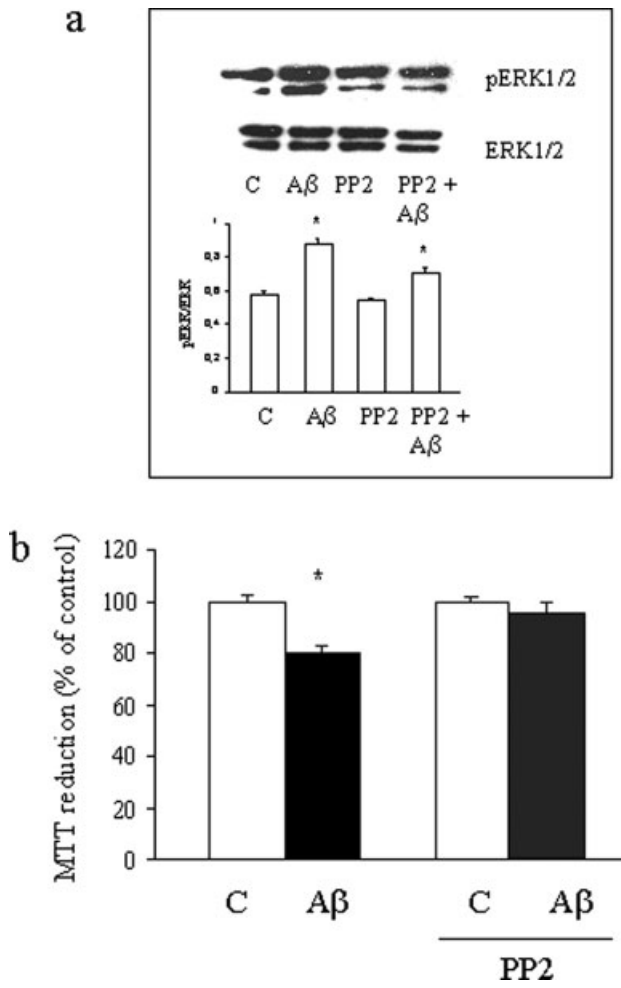

Fig. 3. PP2 reduced ERK1/2 activation and prevented neuronal death induced by Aβ. PP2 (1 μM) was added to the cultures 20 min prior to exposure to $A\beta_{(25–35)}$. **a:** Western blot and densitometric analyses of 4 experiments of ERK1/2 phosphorylation following exposure of differentiated SH-SY5Y cells to 25 μM $A\beta_{(25–35)}$ for 30 min ($^{\star}P < 0.05$ vs. all others). **b:** Cell viability analyzed by MTT assay of differentiated SH-SY5Y cells exposed to 25 μM $A\beta_{(25–35)}$ for 48 hr. Data represent mean ± SEM of three independent experiments ($^{\star}P < 0.05$ vs. control).

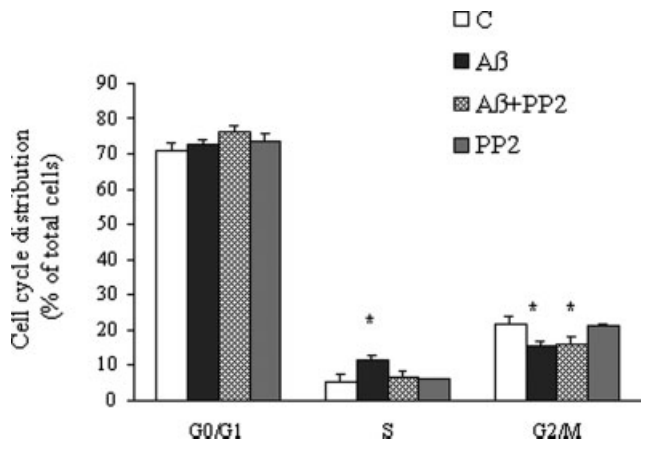

Fig. 4. PP2 prevented Aβ-induced accumulation of cells in S phase. PP2 (1 μM) was added to differentiated SH-SY5Y cultures 20 min prior to exposure to 25 μM $A\beta_{(25–35)}$ for 24 hr. Cell-cycle distribution profiles were analyzed by flow cytometry following propidium iodide staining with the aid of Multicycle AV software. Bars represent means ± SEMs of 3–5 independent determinations ($^{\star}P < 0.05$ vs. respective control).

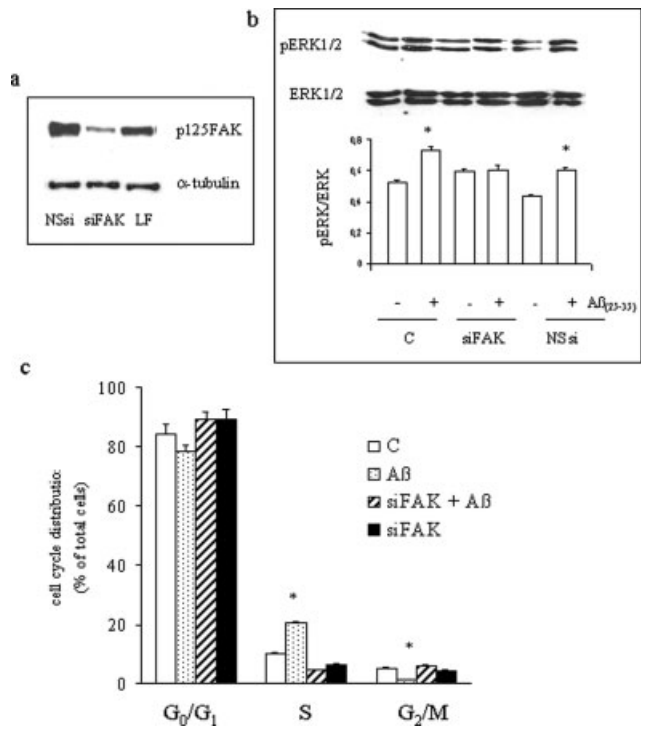

Fig. 5. FAK knockdown prevented Aβ-induced ERK1/2 phosphorylation and cell-cycle activation. **a:** Western blot analysis showing reduced FAK expression 24 hr after transfection of SH-SY5Y cells with FAK siRNA (siFAK) but no significant effect on FAK level of nonspecific siRNA (NSsi) and lipofectamine alone (LF). Induction of pERK1/2 by $A\beta_{(25–35)}$ was abolished in SH-SY5Y cells treated with FAK siRNA. **b:** Representative blot and densitometric analysis of 3 independent studies. **c:** Cell-cycle analysis of cells treated with FAK siRNA showing $A\beta_{(25–35)}$ prevented accumulation of cells in S phase. Data are from 2 independent determinations ($^{\star}P < 0.05$ vs. respective control).

DISCUSSION

The present results identified the interaction of Aβ with integrins as an early step in the sequence of events that links Aβ action to ERK activation, induction of the cell cycle, and neuronal death.

We have previously demonstrated that Aβ is able to induce modifications in cell-cycle progression in differentiated SH-SY5Y cells through the involvement of ERK (Frasca et al., 2004), providing support for the hypothesis that there is a causal relationship among Aβ-induced ERK activation, cell-cycle induction, and neuronal death.

Trying to identify early steps in Aβ-induced cell-cycle induction, attention has been given to integrins

whose role in Aβ effects has been clearly demonstrated, despite some controversies.

In neuroblastoma cell lines, Aβ-induced apoptotic death is accompanied by a strong reduction in the expression of $\alpha_1\beta_1$-integrin. Adhesion of cells on extracellular matrix proteins such as laminin and fibronectin partially rescues neuroblastoma cells from Aβ-induced apoptosis, suggesting that the protective effect of matrix proteins against Aβ toxicity is mediated by integrins. Accordingly, specific antibodies against β_1- and α_1-integrin subunits significantly enhance Aβ-induced apoptosis (Bozzo et al., 2004).

On the other side, β_1-integrin subunit is involved in Aβ-stimulated generation of reactive oxygen species in glial cells (Bamberger et al., 2003), and $\alpha_2\beta_1$ and $\alpha_v\beta_1$ facilitate Aβ deposition and contribute to Aβ neurotoxicity in human cortical neurons (Wright et al., 2007). In addition, the α_1 subunit and the $\alpha_1\beta_1$-integrin heterodimer play an essential role in initiating intracellular signaling events leading to neurite degeneration and cell death in aging hippocampal neurons exposed to Aβ, as demonstrated by the reduced activation of ERK and neuronal death in the presence of α_1- or $\alpha_1\beta_1$-integrin-blocking antibodies (Anderson and Ferreira, 2004).

The SH-SY5Y human neuroblastoma cell line predominantly expresses two integrin complexes, $\alpha_1\beta_1$ and $\alpha_3\beta_1$, and differentiation with retinoic acid leads to a fivefold increase in $\alpha_1\beta_1$-integrin expression, with a specific pronounced increase in the α_1-integrin subunit (Rossino et al., 1991; Meyer et al., 2004). Thus, our attention has been focused on α_1-integrin, and our results are in line with the findings of Anderson and Ferreira (2004) showing that pretreatment of cells with α1-integrin-blocking antibody partially prevented Aβ-induced apoptosis. However, analysis of cell-cycle distribution showed that α1-integrin-blocking antibody per se induced a shift of the cells toward the G2/M phases of the cycle. Under these conditions, the chance to detect a decrease in Aβ-induced effect was remote, so we could not derive any conclusion about the possibility that this treatment was in fact affecting the changes in cell-cycle distribution induced by Aβ. Because of this technical limitation, we concentrated on the first level of signaling downstream of integrin activation. The tyrosine kinase FAK colocalizes with surface integrins and is activated on their binding by specific ligands (Schlaepfer and Hunter, 1996). A role for FAK in Aβ toxicity has been suggested by the reported activation of FAK in both dystrophic neurites surrounding senile plaques and neuronal cultures exposed to Aβ (Zhang et al., 1994; Grace and Busciglio, 2003). In addition, Aβ causes an increased association of Fyn, a member of the Src family, with FAK in rat and human neuronal cultures (Williamson et al., 2002). Src family protein tyrosine kinases colocalize with FAK and are strongly implicated in integrin-mediated effects as well as in the induction and progression through different phases of the cell cycle (Twamley-Stein et al., 1993; Roche et al., 1995). Inhibition of Src kinase family by PP2 prevents ERK2 activation induced by Aβ, suggesting that FAK/Fyn association is upstream of MAPK signaling in Aβ-treated neurons (Williamson et al., 2002). Accordingly, our data show that pretreatment of differentiated SH-SY5Y cells with PP2 reduced activation of ERK1/2 induced by Aβ, an effect that was accompanied by a reduction in Aβ-induced neuronal death and complete prevention of the accumulation of cells in the S phase. In our system, similar results were obtained with genistein, which acts as an aspecific inhibitor of tyrosine kinases at micromolar concentrations (not shown). To confirm further and strengthen a central role for FAK signaling in mediating Aβ action through integrin on one side and ERK1/2 phosphorylation and cell-cycle activation on the other side, effects similar to those obtained with pharmacological inhibition of FAK were observed after the silencing of FAK expression. The use of a specific FAK siRNA produced reduced expression of FAK more than 70% that of the control, a condition that was sufficient to completely prevent increased ERK1/2 phosphorylation and induction of the cell cycle by $A\beta_{(25–35)}$. A slight difference in the two experimental conditions was that although PP2 was not able to modify the reduction in the cell population in the G2/M phase induced by Aβ, causing redistribution of cells in the G0/G1 phase, FAK silencing completely reverted the fall in the G2/M phase induced by $A\beta_{(25–35)}$. Although the reason for this discrepancy is not clear from the present data; it seems consistent with a crucial action of FAK in cell-cycle progression that can be differently modified depending on the level of FAK expression and/or activity. The ability of FAK knockdown to completely prevent ERK1/2 activation agrees well with the observed recovery of cell-cycle distribution profiles as in the control conditions.

In conclusion, our results suggest that neurons challenged with Aβ undergo a specific sequence of events that includes interaction with integrins, activation of FAK/src, ERK1/2, and induction of an unscheduled cell cycle responsible for death. Interestingly, although inhibition of Src kinase family by PP2 completely prevented Aβ-induced cell death, inhibition of ERK activation by PD98059 only partially reduced Aβ toxicity, as in our previous findings (Frasca et al., 2004). Thus, FAK/src may represent a common step of Aβ signaling neuronal death, followed by intervention of multiple pathways that differentially contribute to Aβ toxicity.

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