FRAT1 peptide decreases Aβ production in swAPP$_{751}$ cells

Baolin Li*, John Ryder, Yuan Su, Yan Zhou, Feng Liu, Binhui Ni

Lilly Research Laboratories, Eli Lilly and company, Lilly Corporate Center, Drop code 0510, Indianapolis, IN 46285, USA

Received 24 August 2003; revised 15 September 2003; accepted 15 September 2003

First published online 25 September 2003

Edited by Barry Halliwell

Abstract Recently, LiCl has been shown to inhibit amyloid β peptide secretion in association with diminished glycogen synthase kinase β (GSK3β) activity. However, it remains unclear if direct inhibition of GSK3β activity will result in decreased Aβ production. Frequently rearranged in advanced T-cell lymphomas 1 (FRAT1) protein is a negative regulator of GSK3α/β kinase activity. To examine whether direct inhibition of GSK3α/β kinase activity can lower Aβ production, a FRAT1 peptide was expressed in swAPP$_{751}$ cells that produce high levels of Aβ. Our data demonstrate that cellular expression of FRAT1 peptide in swAPP$_{751}$ cells increases both GSK3α and β phosphorylation on Ser21 and Ser9, respectively, while inhibiting kinase activity of both isoforms. Moreover, as a result of FRAT1 expression, the production of both total Aβ and Aβ$_{1-42}$ was significantly decreased. Thus, we provide evidence that direct regulation of GSK3α/β by FRAT1 peptide significantly decreases Aβ production in swAPP$_{751}$ cells.
© 2003 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.

Key words: FRAT; Glycogen synthase kinase; Amyloid; Amyloid precursor protein; Alzheimer's disease; Kinase activity

1. Introduction

Many believe that the deposition of Aβ in the brain is an early and an underlying event in the development of Alzheimer's disease (AD) [1]. In fact, according to the amyloid cascade hypothesis, the inhibition of Aβ production and accumulation is considered to be a promising therapeutic approach for treatment of this disease. Indeed, many approaches have been proposed to inhibit Aβ production/accumulation including inhibiting β-secretase [2,3] and γ-secretase [4–6], regulating ApoE signaling [7,8] and, more recently, applying Aβ antibody intervention [9–11].

Glycogen synthase kinases GSK3α/β are serine/theronine-specific multifunctional protein kinases that are thought to play an important role in the regulation of a variety of cellular processes in vivo [12–14]. Indeed, GSK3α/β have been implicated in many signal transduction pathways including PI3 signaling [15] and Wnt signaling cascades [16]. Phosphorylation of GSK3α/β at N-terminal serine by kinases, like PI3 activated protein kinase B (Akt/PKB), negatively regulates GSK3α/β kinase activity. However, Wnt signaling results in the inhibition of GSK3β in a PKB independent fashion. As demonstrated in Drosphilia, Wnt inhibits GSK3β in a signaling complex with Dvl, GSK binding protein (GBP), axin and β-catenin [17]. GBP is a positive regulator of Wnt signaling by negatively regulating GSK3β leading to the stability of β-catenin. Frequently rearranged in advanced T-cell lymphomas 1 (FRAT1) is the mammalian homologue to GBP [18]. GSK3β have been implicated in the development of AD pathology [19,20], which has positioned GSK3β as an attractive potential target for treatment of AD. GSK3α implication in AD pathology is largely unknown.

FRATs are GSK3α/β binding proteins, composed of three members, FRAT1, FRAT2 and FRAT3. As components of a Wnt signaling complex, FRAT1 and 2 can physically bind to GSK3β and inhibit its kinase activity [17]. FRAT1 and FRAT2 share a high degree of homology in their C-terminal region [18]. It has been demonstrated that FRAT peptides from the conserved C-terminal region in FRAT1 and 2 are capable of inhibiting GSK3α/β kinase activity by binding to either GSK3α or β [18].

Many lines of evidence have demonstrated that GSK3β is involved in the hyperphosphorylation of tau, a classical pathological feature of AD [21]. In fact, GSK3β is a major contributor to the phosphorylation of tau [21]. GSK3α/β involvement in Aβ production, however, is largely unknown. In order to study effects on APP processing, we expressed a FRAT1 peptide in swAPP$_{751}$ stable cells to inhibit GSK3α/β kinase activity and examined subsequent Aβ production.

2. Materials and methods

2.1. Cell culture

In this investigation, we used well-characterized high Aβ producing HEK293 cells stably transfected with human Swedish APP$_{751}$ [22], denoted as swAPP$_{751}$. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM/F12 3:1; Life Technologies, Gaithersburg, MD, USA) supplemented with 5% fetal bovine serum, 20 mM HEPES, 50 μg/ml tobramycin, and 300 μg/ml G418 and maintained at 37°C and 5% CO_2.

2.2. Construction of FRAT1 peptide expression vector – FRAR1tide

To express a human FRAT1 peptide, the coding DNA sequences for a 39 amino acid peptide from the C-terminal of human FRAT1 were cloned into a pcDNA3.1-V5-His-TOPO vector (Invitrogen, Carlsbad, CA, USA) by employing polymerase chain reaction (PCR) strategies to form an expression vector, designated FRAT1tide hereafter. Briefly, two 75-mer oligos were synthesized. Oligo 1 (5′CACCATGTCCCAACCAGAAACCCGCACAGGCGACGACGACCCGCACCGGCTTCTGCAGCAGCTAGTGCTCTCTGGAAAC-3′) is a FRAT1 sense strand containing a start codon (ATG) and partial FRAT1tide coding sequences of the FRAT1tide N-terminal portion. Oligo 2 (CTGCAGCCGTCGCGAATGAAGCCTTCGCACGGCCTCCTTGATGAGGTTTCCAGAGAGCACTAGCTGCTGCAGAAG-3′) is a FRAT1tide antisense strand containing partial FRAT1tide coding sequences of the FRAT1tide C-terminal portion. Oligos 1

*Corresponding author. Fax: (1)-317-276 5546.
E-mail address: li_baolin@lilly.com (B. Li).

0014-5793/03/$22.00 © 2003 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)01042-1

and 2 contained 30 bases overlapping in the middle of the FRAT1tide coding sequences and served as FRAT1tide PCR templates for annealing and extending at the beginning of PCR. Two additional oligos were synthesized as PCR primers. The forward primer (5′CACCATGTCCCAACCAGAAACCCGCACA-3′) consists of Kozac sequences, a start codon and partial 5′-end coding sequences of FRAT1tide. The reverse primer (5′-CTGCAGCCGTCGCGAATGAAGCCTTCG-3′) covers the 3′-end of FRAT1tide coding sequences. The PCR products were in-framely cloned into pcDNA3.1/V5-His-TOPO (Invitrogen) to form FRAT1tide. The V5-tag and His-tag were included for detecting FRAT1tide expression at the protein level.

2.3. Transfection and Western blot

The FRAT1tide expression vector was transiently transfected into swAPP$_{751}$ cells using lipofectamine plus following manufacturer's instruction (Gibco, Charsbad, CA, USA). Western blots were conducted as described previously [23]. Antibodies used in Western blots against phospho-GSK3α/β, GSK3α/β protein and β-actin were purchased from Cell Signaling Technology (Beverly, MA, USA), Transduction Laboratories (San Diego, CA, USA) and Santa Cruz (Santa Cruz, CA, USA), respectively.

2.4. Aβ enzyme-linked immunosorbent assay (ELISA)

Determination of total Aβ and Aβ$_{1-42}$ was quantified using a sandwich ELISA described previously [24].

2.5. Statistics

All statistical analysis was performed using the Student's *t*-test with a standard *P*-value threshold of ≤ 0.05. Data are presented as mean ± S.D.

3. Results

3.1. Expression of FRAT1tide in the swAPP$_{751}$ cell alters APP processing

The final FRAT1tide expression vector contained the following sequences from 5′ to 3′: a Kozac sequences, a start codon (ATG), coding sequences for FRAT1tide, coding sequences for V5-tag and His-tag and a stop codon (Fig. 1A). The FRAT1tide expression vector was transiently transfected into swAPP$_{751}$ cells. Forty-eight hours after transfection, cells were harvested and the resulting cell lysate was subjected to

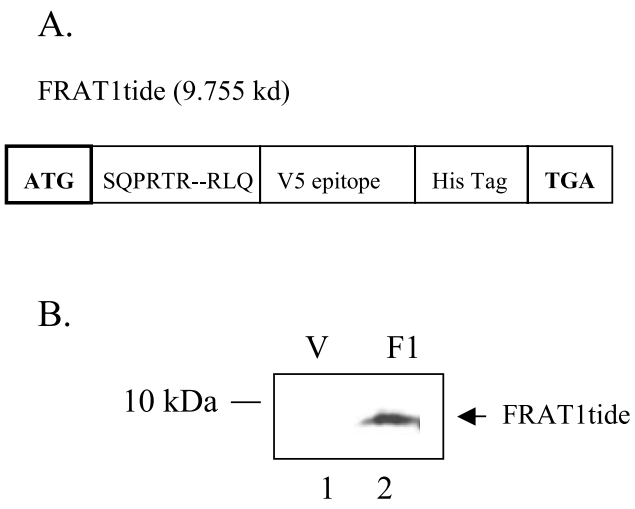

Fig. 1. Structure of FRAT1tide expression vector and Western blot showing the expression of FRAT1tide in swAPP$_{751}$ cells. Panel A shows that the FRAT1tide expression vector consists of a start codon (ATG), 39 amino acid FRAT1 peptide, V5 epitope and His-tag. Panel B is a Western blot showing the expression of FRAT1-tide in swAPP$_{751}$ cells with the predicted molecular weight, 9.7 kDa. Lane 1 represents vector control. Lane 2 represents transfected FRAT1tide.

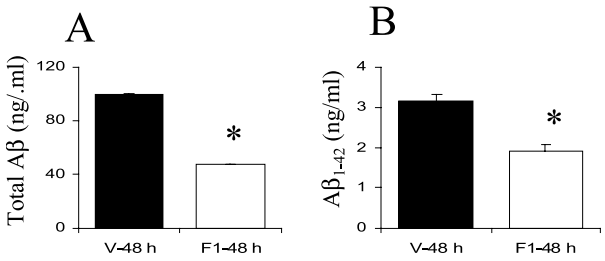

Fig. 2. Expression of FRAT1tide in swAPP$_{751}$ cells significantly decreases levels of both total Aβ and Aβ$_{1-42}$. Results shown represent three independent experiments. In both panel A and B, V and F1 stand for vector and FRAT1tide, respectively. Panel A shows total Aβ levels in the medium harvested 48 h after transfection of vector or FRAT1tide into swAPP$_{751}$ cells. The differences of total Aβ levels between V and F1 are statistically significant ($P < 0.01$). Panel B shows Aβ$_{1-42}$ levels in the medium harvested 48 h after transfection of vector or FRAT1tide into swAPP$_{751}$ cells. The differences of Aβ$_{1-42}$ levels between V and F1 are statistically significant ($P < 0.05$).

Western blot analysis using an HRP-V5 antibody (Invitrogen, Carlsbad, CA, USA). As shown (Fig. 1B), FRAT1tide expression vector coded a HRP-V5 antibody detected peptide at the predicted molecular weight in swAPP$_{751}$ cells.

In this study, the correlation between expression of FRAT1tide and Aβ production was addressed using swAPP$_{751}$ cells. FRAT1tide transfection experiments were carried out for 48 h. Culture medium and cells were then harvested separately. The cell medium was used to examine Aβ production. As shown in Fig. 2, FRAT1tide expression significantly decreased the production of both total Aβ (Fig. 2A) and Aβ$_{1-42}$ (Fig. 2B) in swAPP$_{751}$ cells at 48 h following transfection. Overall, total Aβ production was decreased more than Aβ$_{1-42}$ production. These data suggest that expression of FRAT1tide is sufficient to decrease Aβ production in swAPP$_{751}$ cells. To confirm that the FRAT1tide mediated decrease in Aβ production is not due to FRAT1tide expression related toxicity, swAPP$_{751}$ MTT cell viability assays were also carried out at 48 h following transfection. There were no differences in cell viability between FRAT1tide and vector transfected cells (data not shown).

3.2. FRAT1tide expression in the swAPP$_{751}$ cell negatively modulates GSK3 kinase activity

In order to investigate whether the FRAT1tide expression mediated decrease in Aβ production involved direct inhibition of GSK3α/β kinase activity, GSK3α/β phospho-immunoblot analysis was conducted. swAPP$_{751}$ cells were again transfected with FRAT1tide for 48 h, to correlate with the reduction in Aβ levels described above, harvested and subjected to Western blot analysis with antibodies against phospho-GSK3α on Ser21 and phospho-GSK3β on Ser9, GSK3α/β protein and β-actin. As shown in Fig. 3A, expression of FRAT1tide resulted in elevated phosphorylation on both GSK3α and GSK3β, suggesting negative regulation of these kinases. In contrast, GSK3α and β protein levels were unaffected by FRAT1tide expression (Fig. 3B). β-Actin serves as loading control (Fig. 3C). The relative differences of phosphorylation of GSK3α and β were analyzed by quantitative densitometry (Fig. 3D,E). Since it is known that elevated phosphorylation on Ser21/9 of GSK3α/β is correlated with decreased kinase activity, these data suggest that FRAT1tide inhibits GSK3α/β kinase activity in swAPP$_{751}$ cells.

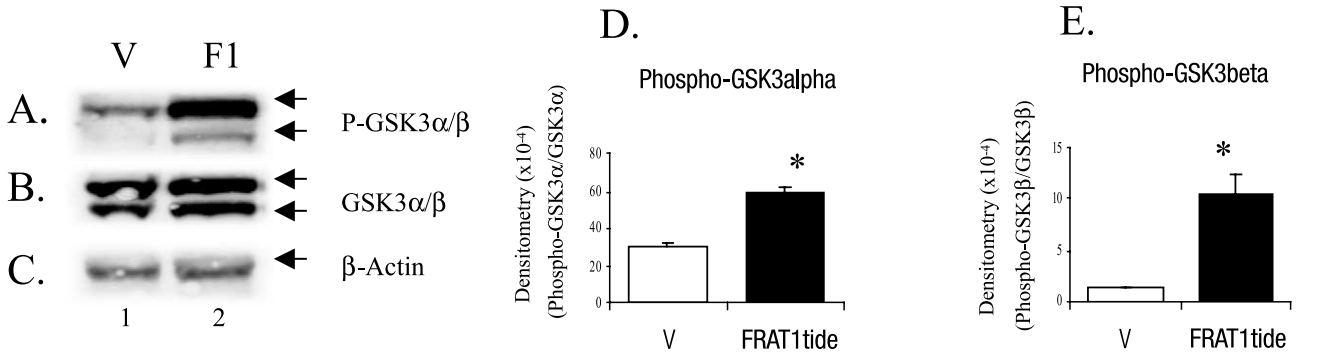

Fig. 3. Expression of FRAT1tide in swAPP$_{751}$ cells directly inhibits GSK3α/β kinase activity measured by increased phosphorylation of both GSK3α and β on ser21 and Ser9, respectively. In all panels, V and F1 stand for vector and FRAT1tide. Panels A–C are Western blots probed with antibodies against phospho-GSK3α/β, GSK3α/β protein and β-actin, respectively. β-Actin serves as loading control. The differences of phospho-GSK3α and β levels between vector and FRAT1 were quantitatively analyzed using densitometry, normalized to GSK3α and β protein levels and are shown in panels D and E, respectively. The results shown in panels D and E represent data from three independent experiments. The differences of phosphorylated GSK3α/β levels between V and FRAT1tide are statistically significant ($P < 0.01$) in both panels D and E.

4. Discussion

The goal of this study was to investigate whether direct inhibition of GSK3α/β kinase activity could lead to a decrease in Aβ production. GSK3β is known to hyperphosphorylate tau and is a component of the wnt signaling cascade where it is negatively regulated in a complex with FRAT1. However, there is little evidence that GSK3α/β is involved in Aβ production. Although a recent study showed that lithium inhibited Aβ production in COS7 cells transiently transfected with APP C100, suggesting that lithium elicits its effect through GSK3β [25], there was no direct evidence to implicate GSK3β. In our studies, however, we used a FRAT1 peptide derived from the highly conserved C-terminal region of human FRAT1 that is known to be a selective GSK3α/β inhibitor [18]. Using swAPP$_{751}$ transfected cells, we provide evidence that direct inhibition of GSK3α/β kinase activity by FRAT1 peptide elicits a decrease in Aβ production.

Moreover, we observed that phosphorylation of both GSK3α on Ser21 and GSK3β on Ser9 was robustly increased by FRAT1tide. This is reasonable because FRAT peptide can physically interact with both GSK3α and β. Interestingly, GSK3α was phosphorylated to a higher degree than GSK3β (more than four-fold) (Fig. 3A), suggesting GSK3α plays an important role in APP processing. Indeed, this result agrees with the recent observation that inhibition of GSK3α kinase activity significantly inhibits Aβ production in vitro [26]. It remains unclear, however, why physically binding of FRAT peptide to GSK3α/β could increase phosphorylation of GSK3α and β. Our hypothesis is that the FRAT-GSK3α/β binding could make the GSK3α/β protein conformation more accessible to be phosphorylated by upstream kinases or less accessible to be dephosphorylated by phosphatases. It is well known that phosphorylation of both GSK3α on Ser21 and GSK3β on Ser9 negatively regulates GSK3α/β activity. However, it is conceivable that the phosphorylated GSK3α/β by FRAT1 peptide may directly participate in the APP processing by forming complexes with several other proteins such as presenilin [27], a component of the gamma secretase complex.

FRAT1 peptide, a sufficient and specific inhibitor for both GSK3α and β kinase activity, provides a valuable tool to further understand the relationship between GSK3α/β signaling and Aβ production. The specific mechanism of how GSK3α/β is involved in Aβ production or perhaps plaque formation warrants further investigation.

Taken together, we conclude that expression of a FRAT1 peptide is sufficient to inhibit GSK3α/β kinase activity in swAPP$_{751}$ cells while significantly lowering Aβ production. This result is interesting, since FRAT peptides can physically bind to GSKα and β. To our knowledge, this is the first report that shows direct inhibition of GSK3α/β kinase activity by FRAT1 peptide results in decreased Aβ production.

Acknowledgements: We would like to thank Dr. Patrick May for critical comments on this paper.

References

[1] Verbeek, M.M., Ruiter, D.J. and de Waal, R.M. (1997) Biol. Chem. 378, 937–950.
[2] Huse, J.T., Bryant, D., Yang, Y., Pijak, D.S., D'Souza, I., Lah, J.J., Lee, V., M.Doms, R.W. and Cook, D.G. (2003) J. Biol. Chem. 27, 27.
[3] Citron, M. (2002) Nat. Neurosci. 5, 1055–1057.
[4] Rochette, M.J. and Murphy, M.P. (2002) Mol. Neurobiol. 26, 81–95.
[5] Grimm, H.S., Beher, D., Lichtenthaler, S.F., Shearman, M.S., Beyreuther, K. and Hartmann, T. (2003) J. Biol. Chem. 29, 29.
[6] Dovey, H.F., John, V., Anderson, J.P., Chen, L.Z., de Saint Andrieu, P., Fang, L.Y., Freedman, S.B., Folmer, B., Goldbach, E., Holsztynska, E.J., Hu, K.L., Johnson-Wood, K.L., Kennedy, S.L., Kholodenko, D., Knops, J.E., Lee, M., Liao, Z., Lieberburg, I.M., Motter, R.N., Mutter, L.C., Nietz, J., Quinn, K.P., Sacchi, K.L., Seubert, P.A., Shopp, G.M., Thorsett, E.D., Tung, J.S., Wu, J., Yang, S., Yin, C.T., Schenk, D.B., May, P.C., Altstiel, L.D., Bender, M.H., Boggs, L.N., Britton, TC., Clemens, J.C., Czilli, D.L., Dieckman-McGinty, D.K., Droste, J.J., Fuson, K.S., Gitter, B.D., Hyslop, P.A., Johnstone, E.M., Li, W.Y., Little, S.P., Mabry, T.E., Miller, F.D. and Audia, J.E. (2001) J. Neurochem. 76, 173–181.
[7] Teter, B., Raber, J., Nathan, B. and Crutcher, K.A. (2002) J. Alzheimer's Dis. 4, 155–163.
[8] Strittmatter, W.J. and Bova Hill, C. (2002) Curr. Opin. Lipidol. 13, 119–123.
[9] Bard, F., Barbour, R., Cannon, C., Carretto, R., Fox, M., Games, D., Guido, T., Hoenow, K., Hu, K., Johnson-Wood, K., Khan, K., Kholodenko, D., Lee, C., Lee, M., Motter, R., Nguyen, M., Reed, A., Schenk, D., Tang, P., Vasquez, N., Seubert, P. and Yednock, T. (2003) Proc. Natl. Acad. Sci. USA 100, 2023–2028.
[10] McLaurin, J., Cecal, R., Kierstead, M.E., Tian, X., Phinney, A.L., Manea, M., French, J.E., Lambermon, M.H., Darabie,

A.A., Brown, M.E., Janus, C., Chishti, M.A., Horne, P., Westaway, D., Fraser, P.E., Mount, H.T., Przybylski, M. and St George-Hyslop, P. (2002) Nat. Med. 8, 1263–1269.
[11] DeMattos, R.B., Bales, K.R., Cummins, D.J., Paul, S.M. and Holtzman, D.M. (2002) Science 295, 2264–2267.
[12] Frame, S. and Cohen, P. (2001) Biochem. J. 359, 1–16.
[13] Grimes, C.A. and Jope, R.S. (2001) Prog. Neurobiol. 65, 391–426.
[14] Kaytor, M.D. and Orr, H.T. (2002) Curr. Opin. Neurobiol. 12, 275–278.
[15] Dierick, H. and Bejsovec, A. (1999) Curr. Top. Dev. Biol. 43, 153–190.
[16] G. Amlak, M., Uddin, S., Mahmud, D., Damacela, I., Lavelle, D., Ahmed, M., van Besien, K. and Wickrema, A. (2002) Biochem. Biophys. Res. Commun. 297, 760–764.
[17] Li, L., Yuan, H., Weaver, C.D., Mao, J., Farr, G.H., 3rdSussman, D.J., Jonkers, J., Kimelman, D. and Wu, D. (1999) EMBO J. 18, 4233–4240.
[18] Thomas, G.M., Frame, S., Goedert, M., Nathke, I., Polakis, P. and Cohen, P. (1999) FEBS Lett. 458, 247–251.
[19] Baum, L., Hansen, L., Masliah, E. and Saitoh, T. (1996) Mol. Chem. Neuropathol. 29, 253–261.
[20] Bhat, R.V. and Budd, S.L. (2002) Neurosignals 11, 251–261.
[21] Sperber, B.R., Leight, S., Goedert, M. and Lee, V.M. (1995) Neurosci. Lett. 197, 149–153.
[22] Seubert, P., Oltersdorf, T., Lee, M.G., Barbour, R., Blomquist, C., Davis, D.L., Bryant, K., Fritz, L.C., Galasko, D. and Thal, L.J. (1993) Nature 361, 260–263.
[23] Liu, F., Su, Y., Li, B., Zhou, Y., Ryder, J., Gonzalez-DeWhitt, P., May, P.C. and Ni, B. (2003) FEBS Lett. 547, 193–196.
[24] Su, Y., Ryder, J. and Ni, B. (2003) FEBS Lett. 546, 407–410.
[25] Sun, X., Sato, S., Murayama, O., Murayama, M., Park, J.M., Yamaguchi, H. and Takashima, A. (2002) Neurosci. Lett. 321, 61–64.
[26] Phiel, C.J., Wilson, C.A., Lee, V.M. and Klein, P.S. (2003) Nature 423, 435–439.
[27] Tesco, G. and Tanzi, R.E. (2000) Ann. NY Acad. Sci. 920, 227–232.