Protein kinase C and F-actin are essential for stimulation of neuronal FAK tyrosine phosphorylation by G-proteins and amyloid beta protein

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Abstract Focal adhesion kinase (FAK) is a protein tyrosine kinase implicated in signal transduction pathways for integrins, neuropeptides, and lysophosphatidic acid. FAK, first discovered in non-neuronal cells, recently has been reported to occur in neurons, where its tyrosine phosphorylation is upregulated by fibronectin and by the Alzheimer's Aβ peptide. The current work has elucidated molecular events leading to tyrosine phosphorylation of FAK in the rat B103 CNS nerve cell line. Activation of receptor-coupled G-proteins by Mas-7 was found to evoke rapid upregulation of FAK tyrosine phosphorylation (Tyr(P)). Upregulation by Mas-7 was blocked by GF109203X, a potent inhibitor of protein kinase C (PKC). Phorbol ester also upregulated FAK-YP, verifying a role for PKC in the transduction cascade. Upregulation of FAK-YP by activation of G-proteins and PKC was dependent upon intact F-actin, as cytochalasin D abolished stimulation by Mas-7 and by phorbol ester. The relatively slow increase in FAK-YP evoked by chronic exposure to Aβ also was abolished by GF109203X and by cytochalasin D. The results show that tyrosine phosphorylation of FAK in neurons is regulated positively by PKC, functioning down-stream from G-proteins through an F-actin-dependent mechanism. The Alzheimer's Aβ peptide is capable of activating elements of this same signal transduction pathway, via membrane events that remain to be determined.

Key words: Focal adhesion kinase; Alzheimer's disease; Protein kinase C; G-protein; Cytoskeleton; Tyrosine phosphorylation

1. Introduction

FAK is an intracellular protein tyrosine kinase that plays a central role in integrin-mediated signal transduction in non-neuronal cells [1]. It is coupled to intricate protein-protein interactions associated with filamentous actin and components of focal adhesions [2]. Tyr(P) of FAK increases its own kinase activity and establishes a target sequence that can be bound by SH2 domains of other lattice-forming signal transduction proteins. Factors found to modulate FAK Tyr(P) now include lysophosphatidic acid, sphingosine, thrombin and various neuropeptides (vasopressin, endothelin, bombesin, and bradykinin) [3,4].

Most work concerning FAK has been done in fibroblasts and platelets. Existence of FAK in the nervous system was first indicated by the measurement of abundant FAK messenger RNA in the brain [5]. The presence of FAK protein recently has been established in nerve cell lines [6,7]. Neuronal FAK is coupled to integrin-dependent neuritogenesis [8] and is developmentally regulated in the CNS [9]. Chronic exposure of rat and human nerve cell lines to the Alzheimer's Aβ peptide causes a non-sensitizing increase in FAK-YP [6]. Aβ-evoked FAK Tyr(P) correlates with neurotoxicity, with respect to dose kinetics, dependence on cell differentiation, and requirement for Aβ aggregation.

Transduction mechanisms that influence FAK Tyr(P) in nerve cells have not been elucidated. In the current study, experiments with the B103 rat CNS nerve cell line show that G-protein activity can elevate FAK-YP through a mechanism dependent upon PKC and on the integrity of F-actin. Upregulation of FAK Tyr(P) by the Alzheimer's Aβ peptide appears to result from an impact of Aβ on elements of this mechanism.

2. Materials and methods

Rat CNS B103 neuroblastoma cells were maintained in DMEM (Gibco/BRL), antibiotics (streptomycin, penicillin, and fungizone, Gibco/BRL), and 10% fetal calf serum (Sigma) in a 6% CO_2 environment. Aβ peptides were synthesized and prepared as described [6]. For experiments with Aβ peptides, cells were plated on 100 mm Falcon tissue culture dishes at a density of 10 000–12,000 cells/cm^2 in a total volume of 4 ml. Cells were grown for 24–48 h and then washed once with warm Hanks' basic salt solution before addition of the aged treatment solutions (DMEM and N_2 supplement of Bottenstein and Sato in place of fetal calf serum) containing Aβ peptides or Me_2SO. For experiments with Mas-7, phorbol ester, and cytochalasin D, B103 cells were cultured at the same density, washed with warm Hanks' buffer, maintained in treatment solution. Agents or control vehicles were added directly to the cultures. After treatment with various agents, cell lysates were obtained and immunoprecipitation and immunoblot carried out with a standard protocol [6]. Briefly, the same amount of total protein from each sample was immunoprecipitated with anti-FAK polyclonal antibody (Upstate Biotechnology, Inc.), immunoprecipitated complexes were resolved with SDS-PAGE, and ECL Western blot was performed with anti-phosphotyrosine antibody RC20H (Transduction Laboratories). For re-probing experiments, membranes were stripped off antibodies by incubation in a solution containing 62.5 mM Tris-HCl, pH 6.7, 2% SDS, and 100 mM β-mercaptoethanol at 55°C for 1–2 h. Then they were blotted with 1:250 anti-FAK monoclonal antibody (Transduction Laboratories). Immunoreactive bands for the anti-FAK mAb were visualized using HRP-conjugated anti-mouse IgG secondary antibody and ECL reagents. Film was analyzed with an LK B scanning densitometer which utilized the GelScan 2 program for integration of peaks. Film images were reproduced using a UMax scanner with the Aldus Photoshop imaging system. Unless specified, all chemicals were obtained from Sigma Chemical Co.

3. Results and discussion

It recently has been reported that G-protein(s) may play a

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Abbreviations: A23187, calcium ionophore; AD, Alzheimer's disease; CNS, central nervous system; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; FAK, focal adhesion kinase; LPA, lysophosphatidic acid; PKC, protein kinase C; PDBu, phorbol ester; Tyr(P) or YP, tyrosine phosphorylation

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role in the stimulatory effect of LPA on FAK Tyr(P) [10]. Our first experiment tested the possibility that FAK Tyr(P) in neuronal cells could be elevated by direct activation of G-proteins. This as well as subsequent experiments employed the B103 nerve cell line, a spontaneously neuritogenic neuroblastoma line cloned from the rat central nervous system [19] which has been utilized previously in studies of FAK Tyr(P) [6]. Levels of FAK Tyr(P) in B103 nerve cells were measured in the presence of increasing doses of Mas-7, a potent G_o and G_i activator. B103 cell cultures maintained in DMEM and N_2 supplements were treated with increasing concentrations of Mas-7 for 1 h. The cells were lysed, and FAK was immunoprecipitated and analyzed with anti-phosphotyrosine ECL Western blot. As shown in Fig. 1A,B, Mas-7 stimulated FAK Tyr(P) in a dose-dependent manner, with half-maximal effect at 1.0 μM. The maximum increase was 3.7-fold.

Effects of G-protein activity in many cases are mediated by downstream actions of protein kinase C. We therefore asked if

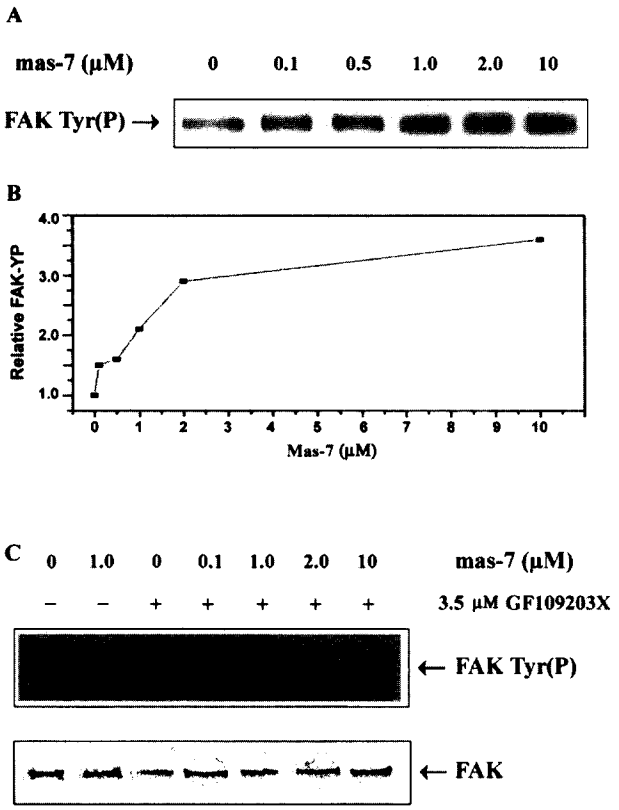

Fig. 1. Mas-7 treatment stimulates FAK Tyr(P) in a PKC-dependent manner. A: B103 cells were cultured in DMEM and N_2 supplement and treated with 0, 0.1, 0.5, 1.0, 2.0, and 10 μM Mas-7 for 1 h. Cells were lysed and FAK Tyr(P) levels were assayed with the standard protocol described in Section 2. The immunoblots show FAK Tyr(P) levels increasing in a dose-dependent manner. B: FAK Tyr(P) levels in A were quantified via densitometry and normalized to controls. The half-maximal response is approximately 1 μM and the increases due to Mas-7 ranged from 1.5- to a maximum of 3.7-fold. C: The dose-response curve of A was repeated in the presence of 3.5 μM GF109203X (indicated by +), a specific inhibitor of protein kinase C. The left two lanes represent controls without GF109203X (indicated by −). The upper panel is a quantitative ECL immunoblot blot of FAK Tyr(P) and shows that GF109203X treatment blocked Mas-7's stimulation of FAK Tyr(P). The lower panel is a re-probe of the same membrane with anti-FAK antibody, and it verifies that equal amounts of FAK protein were present in each sample.

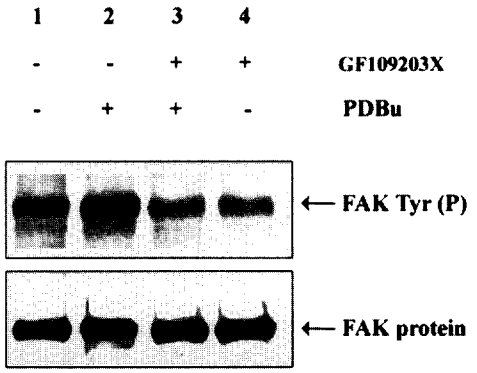

Fig. 2. Phorbol ester activation of protein kinase C enhances FAK Tyr(P). B103 cell samples were treated with either 200 nM phorbol 12,13-dibutyrate (lanes 2 and 3) or the same volume of solvent control (lanes 1 and 4), for 10 min. Two samples (lanes 3 and 4) were simultaneously treated with 3.5 μM GF109203X. FAK protein and tyrosine phosphorylation levels were examined according to standard protocol. The 10 min PDBu treatment increased Tyr(P) levels of FAK 2.3-fold; this effect was blocked by GF109203X. These results demonstrate that PKC, directly or indirectly, can stimulate FAK Tyr(P).

blocking PKC activity might inhibit the increase in FAK Tyr(P) caused by Mas-7. Cells were treated with or without the 3.5 μM GF109203X (bisindolylmaleimide), which has been determined previously to be a selective inhibitor of PKC [11]. As shown in the upper panel of Fig. 1C, Mas-7 failed to stimulate FAK Tyr(P) in the presence of GF109203X. The membrane was stripped and reprobed with anti-FAK mAb. As shown in the lower panel, increases in FAK protein level cannot account for the increases seen in the anti-YP immunoblot. These results indicate that activation of G-proteins can effectively stimulate FAK Tyr(P) in B103 nerve cells, and that this effect is mediated by PKC.

To verify that PKC activity leads to upregulated FAK Tyr(P), we tested whether direct activation of PKC by phorbol esters increased FAK Tyr(P). Cells were treated for 10 min with 200 nM PDBu, a well characterized PKC activator. Control cells were treated with the same volume of chloroform, in which PDBu was dissolved. Equal amounts of cellular lysates were immunoprecipitated with anti-FAK polyclonal antibody, and the immunoprecipitates were analyzed by immunoblotting with the HRP-conjugated anti-phosphotyrosine antibody RC20H. As shown in the upper panel of Fig. 2, PDBu treatment resulted in a marked increase in FAK Tyr(P). Similar results were obtained in 5 other trials (data not shown), with a 2.4 ± 0.2-fold increase in FAK Tyr(P) ($P<0.05$). These results show that direct activation of PKC in B103 cells can stimulate FAK Tyr(P). As would be expected, the increase in Tyr(P) by PDBu was eliminated by simultaneous treatment with 3.5 μM GF109203X (lanes 3 and 4 of Fig. 2). GF109203X kept the Tyr(P) level of FAK close to, or lower than the basal level (compare lanes 1, 3, and 4 in Fig. 2). The lower panel of Fig. 2 shows that recovery of FAK from cell lysates was not altered by treatment with PDBu. Thus increased PKC activity leads to upregulation of FAK Tyr(P) in the B103 CNS nerve cell line. The specific PKC target(s) that leads to increased tyrosine phosphorylation of FAK is not known.

FAK localizes to focal adhesions in non-neuronal cells [12],

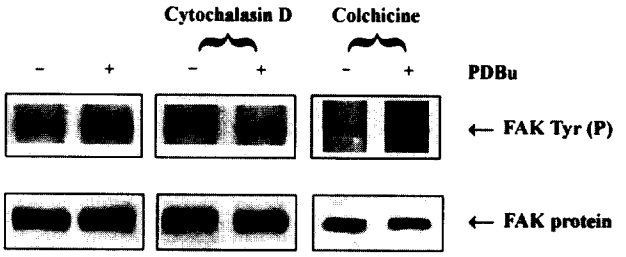

Fig. 3. Actin cytoskeleton network is essential to PDBu's stimulation of FAK Tyr(P). B103 cells were treated with 200 nM PDBu or solvent control for 10 min. In the other two groups of samples, cells were pretreated with 1.0 μM cytochalasin D or 0.5 μM colchicine. FAK protein and tyrosine phosphorylation levels were examined according to standard protocol. Cytochalasin D eliminated Mas-7 stimulation of FAK Tyr(P), while colchicine had no effect. The actin cytoskeletal network thus is required for the stimulation of FAK Tyr(P) by PDBu.

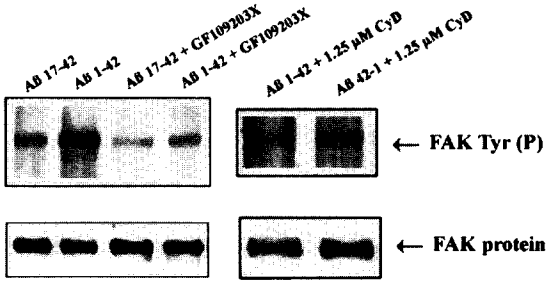

Fig. 5. Stimulation of FAK Tyr(P) by Aβ 1–42 is blocked by GF109203X and cytochalasin D. B103 cells were treated with 20 μM aged Aβ peptides 1–42 or 17–42 for 1 day, with or without 3.5 μM GF109203X or 1.25 μM cytochalasin D. Aβ 1–42 stimulated Tyr(P) levels of FAK 3.4-fold. These effects were blocked by GF109203X and cytochalasin D. These results indicate that Aβ stimulation of FAK Tyr(P) is dependent upon PKC and F-actin.

interacts with actin-binding proteins such as tensin and paxillin [13], and appears to require compartmentalization for activation [14]. These facts suggest that integrity of F-actin networks might be necessary for the stimulation of FAK Tyr(P). We therefore tested the impact of cytochalasin D, which disrupts the actin cytoskeleton [15], on the upregulation of FAK Tyr(P) by PKC activity. B103 cell cultures were pretreated with 1 μM cytochalasin D for 2 h and subsequently treated with 200 nM PDBu for 10 min. As a control, some B103 cell cultures were pretreated for 1 h with 0.5 μM colchicine, which depolymerizes microtubule filaments. The cells were then lysed, and FAK was immunoprecipitated and analyzed with anti-phosphotyrosine ECL Western blot. As shown in Fig. 3, exposure to cytochalasin D eliminated the effect of PDBu on FAK Tyr(P). Although colchicine was observed to evoke neurite retraction, it had no effect on the ability of PDBu to stimulate FAK Tyr(P). The effect of cytochalasin D thus was specific for F-actin and was not due to a general disruption of the cytoskeleton. The immunoblot was stripped off antibody and reprobed with anti-FAK mAb, which showed similar recovery of FAK protein for each sample (lower panels). Cytochalasin D had no effect on cell viability. Analogous inhibition by cytochalasin D was observed for FAK Tyr(P) upregulation caused by Mas-7, as shown in Fig. 4. Pretreatment with colchicine did not alter the effect of Mas-7 (not shown). The F-actin cytoskeleton thus is essential for the G-protein, PKC-dependent upregulation of FAK Tyr(P). The data support a signal transduction model in which F-actin scaffolding coordinates a series of reactions that ultimately lead to tyrosine phosphorylation of FAK.

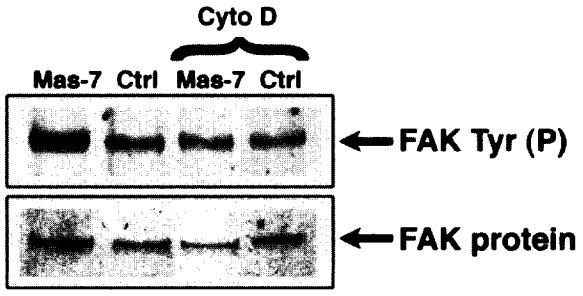

Fig. 4. F-Actin cytoskeletal integrity is required for Mas-7 stimulation of FAK Tyr(P). B103 cells were treated with 1 μM Mas-7 or solvent control for 1 h. In the other two samples, cells were also pretreated with 1.0 μM cytochalasin D (Cyto D) for 2 h. Cytochalasin D treatment eliminated Mas-7 stimulation of FAK Tyr(P), indicating the F-actin cytoskeleton is essential for G-protein upregulation of FAK Tyr(P).

We previously have shown that chronic exposure to the Alzheimer's Aβ peptide also stimulates FAK Tyr(P) in B103 cells. Given the results described above, we therefore examined the potential roles of PKC and the actin cytoskeletal network in mediating the Aβ-stimulated upregulation of FAK Tyr(P). B103 cell cultures were treated with 20 μM aged Aβ peptides 1–42 (neurotoxic), or 17–42 (non-toxic control) for 1 day. The cells were lysed, and FAK was immunoprecipitated and analyzed with anti-phosphotyrosine ECL immunoblot. As shown in Fig. 5 (left panel), Aβ 1–42 treatment markedly increased FAK Tyr(P) in comparison to the control peptide 17–42. To test the role of PKC in stimulation of FAK Tyr(P) by Aβ, 3.5 μM GF109203X was added to cultures. Treatment with GF109203X completely inhibited the effects of Aβ 1–42, with no difference between 1–42 and 17–42 treated samples. Membranes were stripped and blotted with anti-FAK antibody (lower panel), demonstrating that these treatments did not affect recovery of FAK from cell lysates. Similar experiments were performed to test the necessity of actin cytoskeleton for Aβ aggregates' stimulation of FAK Tyr(P). B103 cell samples were treated with either 20 μM Aβ 1–42 aggregates or 20 μM of its reversed form simultaneously with 1.25 μM cytochalasin D for 1 day. The cells were lysed and FAK was immunoprecipitated and analyzed with an anti-phosphotyrosine ECL immunoblot. As shown in Fig. 5 (right panel), cytochalasin D effectively blocked Aβ 1–42 aggregates' stimulation of FAK Tyr(P). Colchicine treatment did not alter the effect of Aβ (not shown). These data show that Aβ stimulates tyrosine phosphorylation of FAK via a mechanism that shares elements of an F-actin-dependent signal transduction pathway. How Aβ triggers the F-actin and PKC-dependent cascade leading to FAK Tyr(P) is unknown. Since Aβ fibrils can attach to cell surfaces [16], one possibility is that membrane perturbations lead to G-protein activation. Promotion of a minor Ca^{2+} leak [17] might theoretically lead to the same response.

Of particular significance is the emerging link between Aβ and F-actin-dependent signal transduction. It now is well recognized that F-actin-dependent signaling plays a central role in regulatory cell biology [2]. Various lines of evidence also

indicate a role for actin-associated signaling in synaptic plasticity [18]. By disrupting multifunctional F-actin-dependent transduction, Aβ could have a profound effect on neurons, giving rise to dementia or memory loss by causing cell death or dysfunction in synaptic plasticity.

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