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Bombesin and angiotensin II rapidly stimulate Src phosphorylation at Tyr-418 in fibroblasts and intestinal epithelial cells through a PP2-insensitive pathway

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Abstract

Src is activated in response to a variety of growth factors and hormones that bind G protein-coupled receptors (GPCRs), and its activity is regulated by phosphorylation at key sites, including the autophosphorylation site Tyr-418 and the inhibitory site Tyr-529. To better understand the mechanisms controlling Src activation, we examined Src phosphorylation in Swiss 3T3 fibroblasts stimulated with bombesin and in IEC-18 intestinal epithelial cells stimulated with angiotensin II (Ang II). Phosphorylation at Src Tyr-418, the activation loop site, was rapidly and markedly increased after GPCR agonist addition in both cell types. However, treatment of intact cells with the selective Src family kinase inhibitor PP2, at concentrations which abolished Src-mediated phosphorylation of focal adhesion kinase (FAK) at Tyr-577, unexpectedly led to increased phosphorylation at Src Tyr-418 and diminished phosphorylation at Tyr-529. In Swiss 3T3 cells, PP2 enhanced Tyr-418 phosphorylation after 1 min of bombesin stimulation, while in IEC-18 cells, PP2 increased Ang II-stimulated Tyr-418 phosphorylation at all times tested. These results imply that a distinct, non-Src family kinase may be responsible for phosphorylating Src at Tyr-418 in intact fibroblasts and epithelial cells stimulated by GPCR agonists.

Keywords: Autophosphorylation; Tyrosine kinase; G protein-coupled receptor; Pyrazolopyrimidine; Intestinal epithelial cells; Fibroblasts; FAK

1. Introduction

The Src family non-receptor tyrosine kinases play important roles in transducing signals in both normal and transformed cells. These kinases are involved in the regulation of multiple cellular processes, including proliferation, motility, adhesion, and differentiation [1–3]. Src has been found to localize to the plasma, perinuclear, and endosomal membranes [1], and in particular, to focal adhesion plaques adjacent to the plasma membrane [4]. Src is activated following binding to integrins, PDGF and EGF receptors, and G protein-coupled receptors [2], and has been implicated in regulating cell motility and proliferation via these receptors [1,5]. Other downstream functions of Src kinases include MAP kinase activation [2], anti-apoptotic signaling [3], cytokine production [6], cytoskeletal remodeling [2], and epithelial permeability [7,8]. In cancer cells, Src mediates anti-apoptotic signals [9] and contributes to transformed phenotypes [10]; it also appears to be overexpressed and highly activated in certain tumors [11,12]. Src and related kinases are therefore important targets for study in both normal and cancerous cells.

The control of Src activity involves multiple intramolecular interactions, regulated by phosphorylation at key sites, which determine whether the molecule will be stabilized in an active or inactive state. The residue Tyr-529 in the C-terminal tail (corresponding to Tyr-527 of chicken Src) is phosphorylated in the inactive state of Src [13,14]. Phosphorylation at Tyr-529 favors binding between the C-terminus and the Src SH2 domain, an interaction that stabilizes the kinase domain in a closed, inhibited conformation [15–17]. The crystal structure of c-Src [18] has shown that the

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SH3 domain makes contact with a helical linker region between the SH2 and kinase domains; this further intramolecular interaction also likely contributes to the suppression of kinase activity [1]. Dephosphorylation of Tyr-529 therefore activates Src by releasing the molecule from its self-bound state. In contrast, Tyr-418 (Tyr-416 of chicken Src) is a positive regulatory site within the Src 'activation loop', a sequence conserved among many tyrosine kinases. When phosphorylated, Tyr-418 is displaced from the substrate-binding site, thus increasing accessibility for Src targets [18,19]. While Tyr-529 is known to be phosphorylated by c-Src c-terminal kinase (Csk), Tyr-418 is generally thought to undergo intermolecular autophosphorylation, a process critically dependent on close proximity of Src molecules [20,21]. However, Tyr-418 does not autophosphorylate rapidly in vitro [20–22], and in one study Src Tyr-418 was suggested as a target for other upstream tyrosine kinases as well [23]. Furthermore, it has been shown that Tyr-418 phosphorylation can enhance Src activity even without dephosphorylation at Tyr-529 [22]. Thus, while the opposing effects of Tyr-529 and Tyr-418 phosphorylation on Src catalytic activity have been extensively investigated, the mechanisms involved are complex and incompletely understood, and likely depend on stimulant and cell context.

Previous results have demonstrated that the mitogenic neuropeptide bombesin, acting through its cognate G protein-coupled receptor (GPCR) in Swiss 3T3 fibroblasts, induces a rapid and transient increase in Src kinase activity and thus plays a critical role in GPCR-induced phosphorylation of focal adhesion kinase (FAK) at Tyr-577 [24,25]. In the present study, we examined the phosphorylation of Src at Tyr-418 in bombesin-stimulated Swiss 3T3 cells, and extended these results using the IEC-18 intestinal epithelial cell line, which expresses the AT1 GPCR for angiotensin II (Ang II). We found that bombesin induced a rapid and transient increase in Src Tyr-418 phosphorylation in Swiss 3T3 cells, while Ang II similarly induced Tyr-418 phosphorylation in IEC-18 cells. To help elucidate the mechanisms of this GPCR-induced phosphorylation, we used the synthetic pyrazolopyrimidine PP2, a well-characterized, potent, and selective inhibitor of Src family kinases which acts by interference with ATP binding [26–28]. Surprisingly, our results show that treatment of intact cells with PP2, at concentrations which completely abolished Src-mediated phosphorylation of FAK at Tyr-577, led to increased phosphorylation at the positive regulatory site Src Tyr-418 and diminished phosphorylation at the negative regulatory site Tyr-529. In Swiss 3T3 cells, PP2 pretreatment increased Tyr-418 phosphorylation after 1 min of cell stimulation, while in IEC-18 cells, PP2 increased Tyr-418 phosphorylation at all times tested. These results imply that in vivo, Src Tyr-418 may be the target of a distinct PP2-insensitive tyrosine kinase which is stimulated by GPCR agonists in both fibroblasts and epithelial cells.

2. Materials and methods

2.1. Cell culture

Swiss 3T3 and IEC-18 cells were purchased from American Type Culture Collection. Stock cultures of these cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) in a humidified atmosphere containing 10% CO_2 and 90% air at 37 °C. For experimental purposes, Swiss 3T3 cells were plated into 100-mm dishes at $4–5 \times 10^5$ cells/dish, in DMEM containing 10% FBS and allowed to grow to confluence (7 days) before use. IEC-18 cells were plated at the same density in DMEM containing 5% FBS and allowed to grow to confluence (4–6 days) before use.

2.2. Lysates and immunoprecipitation

Confluent cultures of 3T3 or IEC-18 cells were washed twice with serum-free DMEM, equilibrated in the same medium at 37 °C for 2 h, then pretreated with or without PP2 or an equivalent volume of solvent for 30 min prior to experimentation. For selected experiments, cells were stimulated by addition to the media of bombesin or Ang II for various times as described in the individual figure legends. Each confluent 100-mm dish contained approximately 3×10^6 3T3 cells, or $4–5 \times 10^6$ IEC-18 cells, in 5 ml of DMEM final assay volume. To prepare whole cell lysates, cell stimulation was terminated by aspirating the medium, adding 400 μl of 2× SDS-PAGE sample buffer (200 mM Tris–HCl, pH 6.8, 2 mM EDTA, 6% SDS, 4% 2-mercaptoethanol, 10% glycerol), scraping immediately on ice, and heating for 20 min at 95 °C. Samples were then resolved in 8% SDS-PAGE gels. For immunoprecipitates, the medium was aspirated and the cells lysed in 1 ml of ice-cold buffer containing 10 mM Tris, pH 7.6, 1% Triton X-100, 50 mM NaCl, 5 mM EDTA, 0.1 mM sodium orthovanadate, 30 mM disodium pyrophosphate, 50 mM NaF, and 1 mM AEBSF (4-(2-aminoethyl)-benzonesulfonyl fluoride HCl; lysis buffer). Lysates were clarified by centrifugation at 15,000 rpm for 10 min at 4 °C, and the pellets were discarded. Proteins were immunoprecipitated overnight at 4 °C with protein A-agarose linked to monoclonal or polyclonal anti-c-Src antibody. Immunoprecipitates were washed two times with lysis buffer and treated further as described for in vitro kinase assay.

2.3. Western blotting

After SDS-PAGE, proteins were transferred to Immobilon-P membranes (Millipore) which were then blocked for 2 h at room temperature with 4% BSA in Tris-buffered saline (TBS) containing 0.1% Tween 20. Membranes were then incubated overnight at 4 °C with anti-SrcY418(P) antibody (0.5 μg/ml), anti-SrcY529(P) antibody (0.5 μg/ml), anti-FAK Y577(P) antibody (0.75 μg/ml), or other antibodies as indicated. The membranes were washed three times with TBS/Tween and