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Extracellular ATP stimulates epithelial cell motility through Pyk2-mediated activation of the EGF receptor

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ABSTRACT

Wounding usually causes considerable cell damage, and released ATP promotes migration of nearby epithelium. ATP binds to purinergic receptors on the cell surface and induces transactivation of the EGF receptor through signaling by the Src family kinases (SFKs). Here we tested whether ATP activates these kinases through Pyk2, a member of the focal adhesion kinase family. Pyk2 was rapidly and potently activated by treating corneal epithelial cells with ATP, and physical interaction of Pyk2 with the SFKs was enhanced. Disruption of Pyk2 signaling either by siRNA or by expression of a dominant-negative mutant led to inhibition of ATP-induced activation of the SFKs and the EGF receptor. Inhibiting Pyk2 activity also blocked ATP stimulation of healing of wounds in epithelial cell sheets. These data suggest that ATP stimulates sequential activation of Pyk2, SFKs, and the EGF receptor to induce cell migration.

1. Introduction

Epithelia respond robustly to damage by migrating and closing wounds in order to regain integrity quickly. Wounding induces many potential stimuli that can promote motility including release of growth factors and cytokines from nearby tissues and blood, exposure of extracellular matrix allowing formation of new interactions with cell surface receptors, mechanical forces, and even the very presence of unconstrained edges in the epithelial cell sheets [1–6] . Wounding invariably involves at least some cell breakage, and released ATP acts as an extracellular signaling molecule, which has been known for some time to induce motility in many cell types [7–11]. ATP exists in high concentrations (low mM) inside cells, and after cell breakage the released ATP binds to purinergic receptors on cells to elicit numerous cellular responses.

Activation of the epidermal growth factor receptor (EGFR) is a common requirement for induction of motility in many cell types after wounding [12–17]. Like many other cues that induce motility, ATP triggers activation of the EGFR through activation of the Src family kinases (SFKs), which leads to proteolytic release of transmembrane ligands such as heparin-binding EGF-like growth factor (HB-EGF) and amphiregulin in a mechanism known as the "triple-membrane-passing" mode of transactivation of the EGFR by G-protein coupled receptors [18–21].

Pyk2 is a member of the focal adhesion kinase (FAK) family [22,23], which are well-established activators of the SFKs. We have previously reported that proline-rich tyrosine kinase 2 (Pyk2) rather than focal adhesion kinase is activated by wounding in conditions that excluded signaling by extracellular ATP [24]. In this paper we examined whether Pyk2 also mediates ATP-induced SFK activation and whether this is necessary for induction of motility by the nucleotide.

2. Materials and methods

2.1. Materials

Antibodies against a C-terminal epitope of the EGFR, the EGFR phosphorylated on tyrosine 1173, Pyk2 phosphorylated on tyrosine 402, FAK phosphorylated on tyrosine 397, and c-Src (mouse monoclonal used for immunoprecipitation) were from Santa Cruz Biotechnology; antibodies against SFK phosphorylated on tyrosine 419, SFK non-phosphorylated on tyrosine 419, c-Src (rabbit polyclonal used for immunoblotting), and the EGFR phosphorylated on tyrosine 845 were from Cell Signaling Technology; antibodies against Pyk2 were from BD Biosciences; antibodies against β-actin were from Sigma. Tyrphostin AG 1478 and Src Kinase Inhibitor-I were from EMD Biosciences. ATP was from Sigma. Cell culture reagents were from MediaTech, and all other supplies and reagents were from ISC BioExpress or ThermoFisher, unless otherwise noted.

Abbreviations: EGFR, epidermal growth factor receptor; SFK, Src family kinase; FAK, focal adhesion kinase; Pyk2, proline-rich tyrosine kinase 2; HCLE, human corneal limbal epithelial; SKI, Src Kinase Inhibitor-I; siRNA, small interfering RNA; PRNK, Pyk2-related non-kinase.

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2.2. Cell culture, ATP treatment, and wounding models

Human corneal limbal epithelial (HCLE) cells have been immortalized by abrogation of p16INK4A/Rb and p53 functions and overexpression of the catalytic subunit of the telomerase holoenzyme [25]. HCLE cells were cultured in keratinocyte serum-free medium (Invitrogen) supplemented with 0.3 mM $CaCl_2$, 25 μg/ml bovine pituitary extract, 0.1 ng/ml human recombinant EGF, 50 IU/ml penicillin, and 50 μg/ml streptomycin. At least 16 h prior to experiments, cells were washed and cultured in the same medium without pituitary extract and EGF. The standard ATP treatment was 5 μM ATP for 5 min. For wound healing assays, cells were cultured to confluence around a single agarose strip [14] and induced to differentiate into a stratified epithelium by culturing in DMEM:F-12 1:1 with 10% newborn calf serum [25]. Three days after transfection with small interfering RNA (siRNA), the agarose strips were removed and cultures were allowed to heal for 18 h before fixation and staining with Gentian violet [14]. Micrographs of cultures were obtained before and after wounding and wound areas were quantified using the region tracing utility in MetaMorph® software (Universal Imaging).

2.3. Western Blotting and immunoprecipitation

Following stimulation, cells were washed in ice-cold phosphate-buffered saline (171 mM NaCl, 10.1 mM Na_2HPO_4, 3.35 mM KCl, and 1.84 mM KH_2PO_4, pH 7.2) and lysed in either SDS (1% in H_2O) or immunoprecipitation buffer (50 mM Tris–Cl, 260 mM NaCl, 0.02% NaN_3, 5 mM EDTA, 1% Triton X-100, 0.5 mM Na_3VO_4, 50 mM NaF, 1 tablet/10 ml of protease inhibitor cocktail (Roche Diagnostics GmbH), and 5 μM pepstatin A, pH 7.5). Protein contents of extracts, determined with the bicinchoninic acid assay (ThermoFisher), were normalized prior to SDS–PAGE. For immunoprecipitation, approximately 250 μg of cellular protein was incubated with 30 μl of protein-A Sepharose slurry and 1 μg of antibody on an end-over-end rotator at 4 °C overnight. Pellets were washed twice with immunoprecipitation buffer and three times with immunoprecipitation buffer with Triton X-100 reduced to 0.1% before addition of SDS–PAGE sample buffer. Multiple exposures of Western Blots were collected, and densitometry of appropriate images was performed using QuantityOne software (BioRad).

2.4. siRNA transfection and adenoviral infection

siRNA duplexes targeting Pyk2 (10 nM) were transfected using siPORT™ NeoFX™ transfection reagent (Applied Biosystems) according to manufacturer's protocol. Cells were then re-seeded after 2 days at experimental densities and used 4 days after transfection. Multiple Pyk2 siRNA oligonucleotides were used: CACAUGAAGUCCGAUGAGAdTdT (Sigma) and a Pyk2 *SMART*pool that contains at least four duplexes of undisclosed sequence (Millipore). As a control siRNA, MISSION® siRNA Universal Negative Control #1 (Sigma) was used. Pyk2 and PRNK cloned into adenovirus vectors were from Dr. Joseph C. Loftus (Mayo Clinic, Scottsdale, AZ). Adenovirus encoding tet-OFF (used as adenovirus control) was from Dr. Ora A. Weisz (University of Pittsburgh, Pittsburgh, PA). For signaling studies, cells were infected at a multiplicity of 10 for 1 h and were used the following day.

2.5. Statistical analysis

All experiments were performed a minimum of three times with similar results. Representative immunoblots are shown below the bar graphs. Densitometry were from at least four replicates, and quantitative data were plotted (means +/− S.D.) and analyzed by *t*-test or one-way ANOVA followed by Bonferroni's multiple comparisons test using Prism (GraphPad Software).

3. Results

3.1. Stimulation with ATP activates Pyk2

We and others have previously reported that extracellular ATP stimulates activation of the EGFR, and that it occurs through a triple-membrane-passing mode of signaling that is mediated by the SFKs [7,11,17]. Members of the focal adhesion kinase (FAK) family are activated by autophosphorylation creating binding sites for the SFKs, which subsequently are activated by another autophosphorylation event. We tested the activation state of FAK with an antibody that we have previously verified recognizes FAK autophosphorylated on tyr-397 in immortalized human corneal limbal epithelial (HCLE) cells [25]. As is illustrated in Fig. 1A no activation was observed after stimulation with ATP. In contrast, strong activation of Pyk2 was readily detected by an antibody that recognizes its major autophosphorylation site on tyr-402. A time course analysis showed detectable activation of Pyk2 up to 2 h after stimulation (Fig. 1B), whereas activation of FAK was not observed at any time point.

Pyk2 can be activated downstream of the EGFR in some systems [26], and we therefore examined the effects of addition of the EGFR kinase inhibitor tyrphostin AG 1478. Although the inhibitor abolished autophosphorylation of the EGFR, no effects were seen on Pyk2 phosphorylation (Fig. 2A). Pyk2 contains tyrosines that can be phosphorylated by the SFKs, and some studies indicate that SFK-mediated phosphorylation regulates Pyk2 activity [27–29]. However, ATP-induced Pyk2 autophosphorylation was unaffected by treatment with the SFK inhibitors Src Kinase Inhibitor-I (SKI) or PP2 (Fig. 2B and C) [30]. The SFK inhibitors blocked EGFR phosphorylation of tyr-845, which is catalyzed by the SFKs [31,32], demonstrating the efficacy of the inhibitors. Together, these results indicate that Pyk2 is activated by exogenous ATP independently of the EGFR and the SFKs.

To determine whether the SFKs are activated by stimulation with ATP in HCLE cells as has been seen in other systems [33–35], extracts were blotted with an antibody that recognizes all isoforms of the SFKs phosphorylated on tyr-419, the major activating autophosphorylation site. This confirmed the expectation that ATP activates SFKs in these cells (Fig. 2D). To determine whether stimulation by extracellular ATP enhances formation of SFK–Pyk2 complexes, we analyzed anti-c-Src immunoprecipitates. We first probed with the phospho-SFK antibody, which showed that the c-Src isoform is activated by addition of extracellular ATP (Fig. 2E). Importantly, increased amounts of Pyk2 were present in the immunoprecipitates of extracts from ATP-treated cells, demonstrating an increased association of c-Src and Pyk2 (Fig. 2F). The interaction was not blocked by the presence of tyrphostin

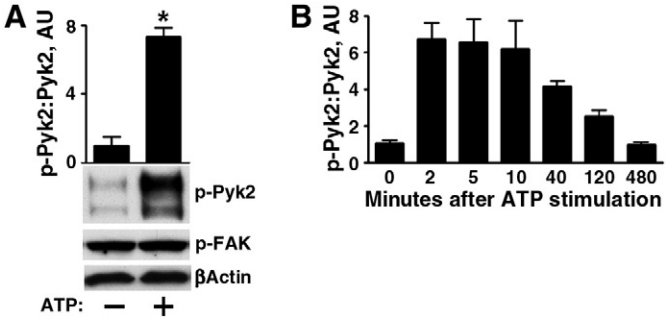

Fig. 1. Activation of Pyk2 by exogenous ATP. (A) HCLE cells were untreated or subjected to the standard treatment with ATP and cell extracts were analyzed by immunoblotting for Pyk2 phosphorylated on tyr-402 (p-Pyk2) or for FAK phosphorylated on tyr-397 (p-FAK). Due to various splicing and post-translational events, the antibody for phosphorylated Pyk2 detects multiple bands, all of which are used in the analysis. The ratio of p-Pyk2:Pyk2 (not shown) was determined by densitometry and * denotes significant differences from untreated cells ($p<0.001$) using the Student's *t*-test. Columns are means and error bars are S.D. in this and the following figures. (B) Time course of Pyk2 activation. AU, arbitrary units.