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Monophosphothreonyl extracellular signal-regulated kinases 1 and 2 (ERK1/2) are formed endogenously in intact cardiac myocytes and are enzymically active

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ABSTRACT

ERK1 and ERK2 (ERK1/2) are central to the regulation of cell division, growth and survival. They are activated by phosphorylation of the Thr- and the Tyr- residues in their Thr-Glu-Tyr activation loops. The dogma is that dually-phosphorylated ERK1/2 constitute the principal activities in intact cells. We previously showed that, in neonatal rat cardiac myocytes, endothelin-1 and phorbol 12-myristate 13-acetate (PMA) powerfully and rapidly (maximal at ~5 min) activate ERK1/2. Here, we show that dually-phosphorylated ERK1/2 rapidly (<2 min) appear in the nucleus following stimulation with endothelin-1. We characterized the active ERK1/2 species in myocytes exposed to endothelin-1 or PMA using MonoQ FPLC. Unexpectedly, two peaks of ERK1 and two peaks of ERK2 activity were resolved using *in vitro* kinase assays. One of each of these represented the dually-phosphorylated species. The other two represented activities for ERK1 or ERK2 which were phosphorylated solely on the Thr- residue. Monophosphothreonyl ERK1/2 represented maximally ~30% of total ERK1/2 activity after stimulation with endothelin-1 or PMA, and their k_{cat} values were estimated to be minimally ~30% of the dually-phosphorylated species. Appearance of monophosphothreonyl ERK1/2 was rapid but delayed in comparison with dually-phosphorylated ERK1/2. Of 10 agonists studied, endothelin-1 and PMA were most effective in terms of ERK1/2 activation and in stimulating the appearance of monophosphothreonyl and dually-phosphorylated ERK1/2. Thus, enzymically active monophosphothreonyl ERK1/2 are formed endogenously following activation of the ERK1/2 cascade and we suggest that monophosphothreonyl ERK1/2 arise by protein tyrosine phosphatase-mediated dephosphorylation of dually-phosphorylated ERK1/2.

1. Introduction

The related prototypic MAPKs, ERK1 and ERK2 (ERK1/2), are Pro-directed Ser-/Thr- protein kinases which regulate cell growth, division and survival through their effects on gene expression and other cellular processes [1–3]. In many dividing cells (including cell lines), growth factor-binding to receptor protein Tyr- kinases leads to activation of ERK1/2 [4,5]. This is also the case in primary cultures of (terminally-differentiated) neonatal rat cardiac myocytes [6,7] but here robust ERK1/2 activation is also induced by Gq protein-coupled receptor-/PKC-dependent pathways [8]. Thus, ligands such as endothelin-1 (ET-1) and tumour-promoting phorbol esters (such as phorbol 12-myristate 13-acetate, PMA) essentially stoicheiometrically activate ERK1/2 in these cells [7].

ERK1/2 are the final components of a protein kinase signalling cascade in which members of the Raf MAPK kinase kinase family (Raf-1, A-Raf and B-Raf) phosphorylate and activate MAPK kinase kinases 1 and 2 (MKK1/2), which in turn phosphorylate and activate ERK1/2 [1]. In all 'classical' MAPKs (ERK1/2, JNKs and p38-MAPKα/β) and some 'novel' MAPKs, activation involves the phosphorylation of a Tyr-residue and a Thr- residue in the activation loop Thr-Xaa-Tyr sequence (Thr-Glu-Tyr in ERK1/2) to produce the dually-phosphorylated MAPK species [1]. In mouse or rat, the relevant residues are Thr-203 and Tyr-205 in ERK1, and Thr-183 and Tyr-185 in ERK2. The general belief is that phosphorylation of both Tyr- and Thr- is necessary for activation of ERK1/2.

Abbreviations: AdV, adenovirus or adenoviral; ET-1, endothelin-1; FPLC, fast protein liquid chromatography; MBP, myelin basic protein; MKK, MAPK kinase; PMA, phorbol 12-myristate 13-acetate; PTP, protein Tyr-phosphatase; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

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Activated ERK1 and ERK2 from cardiac myocytes exposed to ET-1 or PMA have been separated into two discrete peaks of activity by MonoQ Fast Protein Liquid Chromatography (FPLC) with ERK2 eluting at a lower NaCl concentration [6,9]. We show here that each of these peaks of ERK1 or ERK2 activity can be resolved further into two separate peaks. One of these two resolved peaks of ERK1 or ERK2 is dually-phosphorylated [(pT-E-pY)ERK1/2], whereas the second peak is ERK1 or ERK2 monophosphosphorylated on the Thr- residue [(pT-E-Y)ERK1/2]. The important conclusion is that monophosphothreonyl-ERK1/2 can be formed endogenously in cells and contribute significantly to overall ERK1/2 activity.

2. Materials and methods

2.1. Antibodies

The antibody against total ERK1/2 was a rabbit polyclonal from Cell Signaling Technology Inc. (cat. no. 9102). (pT-E-pY)ERK1/2 were detected with a Sigma-Aldrich UK mouse monoclonal antibody (cat. no. M9692) or a Cell Signaling Technology Inc. rabbit monoclonal antibody (cat. no. 4377), as specified in the figure legends. The mouse monoclonal antibodies against (pT-E-Y)ERK1/2 (cat. no. M3557, ERK-YNP, clone 155 in Ref. [10]) and (T-E-pY)ERK1/2 (cat. no. M3682, clone 193 in Ref. [10]) were from Sigma-Aldrich UK. The rabbit anti-FLAG® antibody was from Santa Cruz Biotechnology Inc. (cat. no. sc-7787). Horseradish peroxidase-conjugated secondary antibodies to mouse immunoglobulins (cat. no. P0260) and to rabbit immunoglobulins (cat. no. P0448) were from Dako. The Alexa Fluor®488-conjugated secondary antibody to mouse immunoglogulins (cat. no. A11001) and Texas Red-phalloidin (cat. no. T7471) were from Invitrogen.

2.2. Cardiac myocyte isolation and culture

Primary cultures of ventricular cardiac myocytes were prepared from 1- to 2-day-old Sprague–Dawley rats by an adaptation of the method of Iwaki et al. [11] as described elsewhere [12]. Hearts were removed, ventricles were dissected away from atria, and ventricular myocytes were dissociated by serial digestion with 0.4 mg/ml collagenase and 0.6 mg/ml pancreatin in sterile digestion buffer (116 mM NaCl, 20 mM HEPES, 0.8 mM Na_2HPO_4, 5.6 mM glucose, 5.4 mM KCl and 0.8 mM $MgSO_4$, pH 7.35). The first digestion supernatant (5 min, 37 °C, 160 cycles/min in a shaking water bath) was removed and discarded. Cell suspensions from subsequent digestions (20 min, 2×25 min, 20 min, 10 min; 37 °C, 136 cycles/min shaking) were recovered by centrifugation (5 min, 60×*g*) and the cell pellets resuspended in plating medium [Dulbecco's modified Eagle's medium/medium 199 (4:1 v/v), 15% (v/v) foetal calf serum, 100 units/ml of both penicillin and streptomycin]. The cells were pre-plated on plastic Primaria tissue culture dishes (30 min). The non-cardiomyocytes adhere to the dishes whereas the cardiac myocytes remain unattached. For biochemistry experiments, the non-adherent viable cardiac myocytes from the pre-plates were plated at a density of 4×10^6 cells/dish on 60 mm Primaria dishes pre-coated at room temperature with sterile 1% (w/v) gelatin (Sigma-Aldrich UK). For immunostaining experiments, cardiac myocytes were plated at 2×10^6 cells/dish on 35 mm Primaria dishes containing glass coverslips pre-coated with gelatin as above followed by laminin (Sigma-Aldrich UK, 20 μg/ml in PBS). After 18 h, myocytes were confluent and beating spontaneously. Unless they were to be infected with adenovirus (AdV), serum was then withdrawn and myocytes were incubated in maintenance medium [Dulbecco's modified Eagle's medium/medium 199 (4:1 (v/v), 100 units/ml of both penicillin and streptomycin] for a further 24 h.

2.3. Immunofluorescence microscopy

Cardiac myocytes were exposed to ET-1 (100 nM), then washed 3× with PBS and fixed in 4% (v/v) formaldehyde (10 min, room temperature). Following permeabilisation with 0.1% (v/v) Triton X-100 (10 min, room temperature), non-specific binding was blocked with 2.5% (w/v) bovine serum albumin in 0.1% (v/v) Triton X-100 (10 min, room temperature). Antibodies and fluorophores were diluted in PBS, all incubations were at 37 °C, and coverslips were washed three times in PBS after each stage of the immunostaining procedure. Myocytes were stained with mouse primary monoclonal antibodies to (pT-E-pY)ERKs (Sigma M9692) (1:80, 60 min) and Alexa Fluor®488-conjugated secondary antibodies (1:200, 60 min). Myofilamentous actin was counterstained with Texas Red-phalloidin (5 U/ml, 20 min) and nuclei were counterstained with Hoechst 33258 (10 μg/ml, 15 min). Fluorescence microscopy was performed using a Zeiss Axio Observer Z1 inverted microscope equipped with a Zeiss LSM710 confocal imaging system, and using a Plan-Apochromat 63×/1.4 oil-immersion objective. Standard excitation and emission presets were chosen for Hoechst 33258, Alexa 488, and Texas Red dyes, which were imaged sequentially to eliminate fluorescence bleed-through. All fields were captured at a resolution of 512×512 pixels using a zoom factor of 1.2× with a field dimension of 112×112 μm.

2.4. AdV vector for FLAG-ERK2

The AdV-FLAG-ERK2 vector was prepared using the AdEasy™ XL Adenoviral Vector System (Stratagene) using the general methodology described previously [13]. The FLAG sequence (placed 3′ to an initiation codon) was introduced into the pShuttle-CMV vector and the mouse ERK2 coding sequence was placed in frame and 3′ to the FLAG tag. The FLAG sequence was first introduced between the BglII and KpnI restriction sites in the multiple cloning site of the pShuttle-CMV vector by synthesis of an oligonucleotide cassette containing the FLAG sequence preceded by an ATG start codon. The complementary oligonucleotides used were:

Forward 5′-GATCTACCATG**GACTACAAAGACGATGACGACAAG**GGTACCGTCGACGC-3′;
Reverse 5′-GGCCGCGTCGACGGTACC*CTTGTCGTCATCGTCTTTGTAGTC*CATGGTA-3′

with the FLAG sequence underlined and emboldened, and its antisense complement underlined and italicized. The oligonucleotides were annealed and phosphorylated, resulting in a cassette flanked by 'cut' BglII and NotI sites, which was then inserted into the corresponding restriction sites in the multiple cloning site of pShuttle-CMV. This strategy, which replaces the KpnI–NotI fragment of pShuttle-CMV with an identical sequence in the cassette insert, was used to avoid the potential difficulty of cutting both the BglII and KpnI sites which lie immediately adjacent to each other in pShuttle-CMV. The mouse ERK2 coding sequence was amplified from a glutathione-*S*-transferase-ERK2 fusion construct in pGEX4T-1 (gift of Professor Chris Marshall, Institute of Cancer Research, London) with Pfu polymerase using forward primer: (5′-GACGACAAG**GGTAC-C**ATGGCGGCCGCAGCAGCGGCCGGC-3′) and reverse primer (5′-CTTATCTAG**AAGCTT**TTAAGATCTGTATCCTGGCTGGAATCTAGCAG-3′) to produce ERK2 flanked by KpnI and HindIII sites (underlined and emboldened) which were then used for insertion into the corresponding sites in FLAG-pShuttle-CMV, 3′ and in frame with the FLAG sequence. All constructs were sequenced on an ABI3730xl or ABI3130xl automated fluorescent DNA sequencer at the Clinical Sciences Centre Genomics Core Laboratory (Imperial College London). Shuttle plasmids containing verified sequences were then linearised with PmeI and used to transform BJ5183-AD-1 cells which were subsequently screened for homologous recombination. AdV plasmids from positive recombinants were expanded in XL10Gold cells, linearised with PacI and used to transform HEK293 cells, as described in the AdEasy XL Adenoviral Vector System manual. Viruses were