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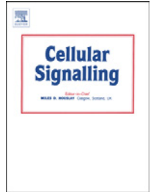

Compartmentalized CDK2 is connected with SHP-1 and β-catenin and regulates insulin internalization

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ABSTRACT

The cyclin-dependant kinase Cdk2 is compartmentalized in endosomes but its role is poorly understood. Here we show that Cdk2 present in hepatic endosome fractions is strictly located in a Triton X-100-resistant environment. The endosomal Cdk2 was found to be associated with the protein tyrosine phosphatase SHP-1, a regulator of insulin clearance, and the actin anchor β-catenin, a known substrate for both Cdk2 and SHP-1. In the plasma membranes and endosome fractions, β-catenin is associated with CEACAM1, also known as regulator of insulin clearance. We show that β-catenin, not CEACAM1, is a substrate for Cdk2. Partial down-modulation of Cdk2 in HEK293 cells increased the rate of insulin internalization. These findings reveal that Cdk2 functions, at least in part, via a Cdk2/SHP-1/β-catenin/CEACAM1 axis, and show for the first time that Cdk2 has the capacity to regulate insulin internalization.

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1. Introduction

Control of the mammalian cell cycle involves sequential activation of cyclin-dependant kinases (Cdks) [1,2]. The role of Cdks in the control of vesicular traffic has long been suspected to account for the reorganization of membranes seen during mitosis [3]. Several cellular activities that are induced by Cdks have been reconstituted, including inhibition by starfish Cdk1 of the formation of hybrid early endosomes [4]. Cdk1, when associated with cyclin A or B, exhibits different *in vitro* phosphorylation profiles in early endosomes; its association with type B cyclin is predicted to be the physiologically relevant one [5]. During the G2 and mitotic phases of the cell cycle, Rab4, a target of Cdk2, is complexed with cyclin B, which regulates endosomal transport to the apical membrane [6,7]. Phosphorylation of Rab4 by Cdk2 leads to its re-localization (translocation) from the endosomes to the cytosol [8,9]. In the adult rat liver, an active Cdk2/cyclin-E pool is compartmentalized in the plasma membrane (PM) and endosomal fractions [10]. It has been shown that Cdk2 associates with the protein tyrosine phosphatase (PTP) SHP-1 (also known as PTP1C, SHPTP-1 or HCP) in human ovarian cancer cells [11] and enterocytes [12], and it was inferred that SHP-1 regulates the cellular compartmentalization of Cdk2 [13]. In this study, we have further characterized the role of Cdk2 with regard to compartmentalized Cdk2/SHP-1 complexes.

2. Experimental procedures

2.1. Reagents and antibodies

Porcine insulin was obtained from Sigma (St. Louis, MO). The antibody directed against the β-subunit of the insulin receptor (IR) was obtained from BD Transduction Laboratories (rabbit polyclonal, 188430). The monoclonal anti-phosphotyrosine (PY) antibody (PY20) was obtained from Sigma Chemicals (St Louis, Mo). The anti-Cdk2, anti-cyclin E and β-catenin antibodies were from Santa Cruz Laboratories (sc-163AC, sc-481AC and sc-7199). The anti-CEACAM1 and anti-SHP-1 antibodies were produced and used as described [14]. For Western blot studies, we used the enhanced chemiluminescence kit, Western Plus (Perkin Elmer Life Sciences Inc., Boston, MA), and Immobilon-P transfer membranes (Millipore, Bedford MA). Reagents for SDS-PAGE were obtained from Bio-Rad (Mississauga, Ontario). All other chemicals were of analytical grade and were purchased from either Fisher Scientific (Sainte-Foy, Québec) or Roche Laboratories (Laval, Québec).

Abbreviations: Cdk, cyclin-dependant kinase; IR, insulin receptor; PM, plasma membrane; G/E, Golgi/endosomes; PTP, protein tyrosine phosphatases; DNSHP-1, Dominant negative SHP-1; HEK, Human embryonic kidney; IIA, Insulin internalization assay; WGL, Wheat germ lectin; LC-MS, Liquid chromatography- mass spectrometry; bpV (phen), bisperoxovanadium 1,10-phenanthroline; i.p., intraperitoneal.

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2.2. Hepatic fractions

Harlan Sprague–Dawley rats (female 120–140 g, body weight) were purchased from Charles River Ltd. (St. Constant, Québec, Canada). Experiments were approved by the Laval University Animal Care Committee. Animals were fasted for 16 h and anesthetized with Isoflurane (Aerrane, Janssen, Ontario). When indicated, an intraperitoneal (i.p.) injection of bisperoxovanadium 1,10-phenanthroline (bpV(phen)) (0.5 mg/150 g, body weight), synthesized as described in [15], occurred 16 h and 30 min before the injection of insulin (1.5 or 15 μg/100 g, body weight, jugular vein) (porcine insulin, Sigma-Aldrich Corporation, MO). The animals were sacrificed by decapitation at times noted after insulin injection (0–60 min). The livers were then excised rapidly and minced before homogenization. The combined fraction of Golgi and endosomes (G/E) elements was prepared immediately, as described in [16]. The liver weight of the rats was 4.9 ± 0.1 g ($n = 56$). The yield of the G/E fraction was 0.47 ± 0.06 mg of protein/g of liver weight ($n = 49$). The microsomal fraction was obtained by resuspending the combined light mitochondrial and microsomal (L + P) fractions in ice-cold homogenization buffer (1.15 M sucrose) [16]. A cytosolic fraction was generated by centrifuging the homogenate at 100,000 *g* for 1 h. The supernatant was collected (5 ml/g of liver; 16 ± 0.9 mg of protein/ml, $n = 36$) and used immediately. A PM fraction was prepared as previously described [17] with minor modifications [18] and used directly. A yield of 1.86 ± 0.13 mg of protein/g of liver ($n = 48$) was obtained. The protein content of each fraction was determined by a modification of the Bradford method using bovine serum albumin as a standard (Bio-Rad protein assay, Bio-Rad, Hercules, CA). Adenovirus preparation and injection for the purification of total mouse dominant-negative SHP-1 (DNSHP-1) hepatic membrane were done as described [14].

2.3. RNAi and insulin internalization assay (IIA)

To downregulate Cdk2, we used the following Invitrogen-predesigned siRNA: CCAGTCTAACTCTGTGTGCTACGCCA. HEK293 cells were cultivated in DMEM-high glucose medium with 10% fetal bovine serum (Hyclone) without antibiotics. Cells were transfected using Lipofectamine™ (Invitrogen) for 48 h and subjected to the immunoblot procedures or the IIA as previously described [14].

2.4. Phosphorylation assays

Phosphorylation of Cdk2 immunocomplexes was performed on G/E fractions that were incubated for 15 min at 37 °C in the presence of 25 μM [γ-^{32}P]ATP (3000 Ci/mmol) [19]. The reaction was stopped by adding ice-cold solubilization buffer. The samples were solubilized for 1 h, diluted (1:2 v/v) with ice-cold buffer and clarified by centrifugation (240,000 *g*, 15 min, 4 °C). The supernatant was subjected to immunoprecipitation using an anti-Cdk2 antibody. The resulting immunocomplexes were subjected to SDS-PAGE, KOH treatment when indicated, and autoradiography. Cdk2 kinase assays were performed as described [20] except that freshly isolated Cdk2 was prepared from HEK293 cells homogenates and the substrate Histone H1, used a positive control, was replaced by affinity purified β-catenin or the bacterially expressed (pGEX-2T, GE-Healthcare PQ) wild type or mutant (Ser503Ala) cytosolic domain of human CEACAM1 (residues: 448–519).

2.5. Sequential immunoprecipitation and mass spectrometry

Glycoproteins were recovered from the wheat germ lectin (WGL) subfraction of the G/E fractions, which had been solubilized (Empigen BB 0.3%, 2 h, 4 °C), clarified as previously described and diluted (8 mg protein) [10]. The eluates were then submitted to sequential immunoprecipitations with antibodies against Cdk2, cyclin-E, SHP-1 and IR β-subunit before proceeding. The immunoprecipitated proteins were analyzed by SDS-PAGE. Bands that were stained with SYPRO Ruby (Bio-Rad, Hercules, CA) were excised from the gel and subjected to tryptic digestion. The resulting peptides were separated using a capillary high-performance liquid chromatography (HPLC) reverse phase C18 column (Picofrit BioBasic, NewObjective, 10 cm length, 0.075 mm internal diameter) and analyzed by tandem mass spectrometry using a LC-MS/MS quadrupole ion trap mass spectrometer (LCQ Deca XP, ThermoFinnigan). The non-redundant NCBI database was searched using Mascot (Matrix Science Inc.).

2.6. Statistical analysis

All data are presented as mean ± standard deviation (s.d.), with the number of experiments in parentheses. The statistical significance was assessed using the two-tailed unpaired Student's *t* tests, with an alpha level of 5% and $P < 0.05$. Two-way analysis of variance (ANOVA) with repetition was used for the statistical analysis over the time course for the IIA assay.

2.7. Supplementary data

Additional detailed methods for electron microscopy, mouse cell fractions, immuno-enriched endosomes are available in the Supplementary Information.

3. Results

3.1. Presence of Cdk2 in a detergent-resistant fraction

There is a paucity of data concerning endosomes-compartmentalized Cdk2 [10]. Following solubilization of rat liver G/E membranes (Triton X-100, 1%), we found Cdk2 present solely in the Triton X-100-resistant pellet, thus precluding analysis of solubilized Cdk2 complexes on our first attempts (Fig. 1A, T-1 lane). We tested a battery of detergents and obtained a complete partition of Cdk2 in the soluble phase with Empigen BB. It was efficient at concentrations of 0.3% and 1.0% (Fig. 1A, Emp lanes). SHP-1, a Cdk2 partner [11], was equally distributed between the Triton X-100-soluble and -insoluble materials (Fig. 1A, T-1 lane). We detected the largest pool of SHP-1 in the hepatic cytosolic fraction (approximately 70% of the total), with consistent pools indeed present in the PM (20%) and G/E (10%) fractions under basal conditions (Fig. 1B). Following acute and subsaturating stimulation with insulin (1.5 μg/100 g, body weight), the IR rapidly accumulated in the endosomes, with a peak accumulation at 2 to 5 min post-injection, as originally observed [16]. Under these circumstances, the levels of SHP-1, SHP-2 and the transmembrane PTP receptor protein tyrosine phosphatase α (RPTPα) were not altered. A marked increase was however measured in both SHP-1 and SHP-2, but the RPTPα levels were unchanged after treatment with the PTP inhibitor, bpV(phen) (Fig. 1C). In particular, SHP-1 levels were significantly increased (>50%) over those of the controls (ratio of bpV(phen)/control, at time 0: 2.1 ± 0.6, $P < 0.05$, $n = 3$, unpaired *t* test) as well as at other times. Use of a ten-fold higher saturating dose of insulin (15 μg/100 g, body weight) did not further change the SHP-1 levels (Fig. 1D). Therefore, both Cdk2 and SHP-1 were present in the Triton X-100 insoluble materials. SHP-1 accumulation was not observed after acute stimulation with insulin. Levels of compartmentalized SHP-1 increased after stimulation with a PTP inhibitor supporting the idea that accumulation of phosphorylated tyrosine residues is important.

3.2. Presence of Cdk2/SHP-1 complexes in liver fractions

Cdk2 was then immunoprecipitated from the solubilized G/E fraction, and SHP-1 association was indeed detected. The complexes were consistently detected with 0.3%, but not 1.0%, Empigen BB, indicating that they are prone to dissociation. Cdk2/SHP-1 complex

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