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Association of Cyclin-Dependent Kinase-4 and Cyclin D1 in Neonatal β Cells after Mitogenic Stimulation by Lysophosphatidic Acid

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Neonatal pancreatic islet β cells retain a mitogenic capacity in response to growth factors. In this study an increased incorporation of 5-bromo-2′-deoxyuridine in response to oleoyl-lysophosphatidic acid is preceded by a GTP-dependent increase in phosphorylation of the extracellular signal-related kinase, ERK1. The presence of cyclin-dependent kinase-4 and an association with a catalytic partner cyclin D1, a process by which the progression through the cell cycle is regulated in other cell types, was shown to follow this. The mechanisms linking ERK1 phosphorylation and activation of cell cycle progression are not known. Investigation of this process in neonatal β cells may provide a common pathway for the early response to growth factors and the conditions required for an increase in β cell mass by proliferation.

There is general agreement that fetal and neonatal pancreatic β cells maintain a proliferative potential which is not generally available to adult β cells. [reviewed 1]. There are a number of conditions, however, under which the adult β cell mass can be increased by replication. These include proliferation of the ductal epithelium and precursor cells of remnant adult pancreas following pancreatectomy [2], and the hyperplasia found in adult β cells in pregnancy [3], or in the obese (C57 BI/6-ob/ob) mouse, either untreated [4] or in response to co-expression of c-myc plus c-Ha-ras oncogenes [5]. Adult islet cells retain a degree of DNA replication, albeit considerably reduced over that seen in fetal and neonatal life where replication is seen in response to glucose and a number of growth factors and growth promoters [1]. In recent studies of neonatal β cell division following prolactin treatment [6] and of the regeneration of intra- and extra-islet β cells which follows neonatal β cell destruction with streptozotocin [7] it was established that pre-existing intra-islet β cells are capable of reentering the cell cycle. In most, if not all, of these studies cell cycle rentry was determined as progression through the G1-S phase boundary of the cell cycle measured by bromodeoxyuridine (BrdU) labelling index, tritiated thymidine uptake or the determination of thymidine kinase activity. An understanding of the earlier stages of G1 progression in the β cell would indicate the steps which lead to this commitment.

It is now understood that the cell division cycle is regulated by a family of protein kinases in which catalytic cyclin-dependent kinases (CDKs) and regulatory cyclin subunits associate and are activated by CDK-activating kinases in a cell cycle-dependent manner [reviewed 8,9]. In other cell types, but not yet shown for the pancreatic β cell, growth factor stimulation of quiescent cells induces D-type cyclin synthesis early in the G1 phase of the cell cycle and the formation of a CDK4-cyclin D complex regulates the subsequent phosphorylation of the retinoblastoma tumor suppressor protein (Rb), allowing release of sequestered transcription factors essential for progression to S phase [8]. Although both the synthesis and assembly of D-type cyclins and CDK4 depend on growth factor stimulation [10], regulation of the catalytic activity is predominantly post-translational. In response to intra- and extracellular signals there is tight regulation of this process by complexes of CDK4 (p36), cyclin D (p33), proliferating cell nuclear antigen (PCNA, p36) and specific inhibitory proteins (p16, p21 and p27) [8,9,11]. A consensus from many studies is that

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cyclin D is a critical component of the proliferative signal provided by growth factors, including serum, during G1.

It is now considered that a major growth factor in serum is lysophosphatidic acid (LPA), a platelet-derived serum phospholipid with multiple cellular effects including stimulation of cell proliferation [12]. We have shown previously that LPA, which may be derived also from islet phospholipid hydrolysis, promotes neonatal β cell mitogenesis and an increased GTP association with p21ras [13]. In fibroblastic cells LPA has been shown to stimulate mitogen-activated protein kinases/extracellular signal-regulated kinases (MAPKs/ERKs) via a guanine nucleotide-coupled pathway requiring p21ras (and p74raf-1) [14]. In studies using an inducible ras expression vector, a causal role for activated ras in the expression of a cyclin D (cyclin D1) could be shown but no mechanism was proposed [15]. However, it can be shown, in a transfected rat-1 cell line, that the product of the early response gene c-myc is involved in a regulatory network sufficient for cell cycle G0/G1 to S phase transition, with transcription of cyclin D1 as the earliest detectable step [16]. As it may be postulated that ras-regulated gene expression during early G1 may follow activation of transcription factors by ERKs [14] we investigated ERK phosphorylation and CDK4/cyclinD1 association in response to LPA in serum-deprived neonatal β cells.

METHODS

[^{35}S]methionine, [^{32}P]orthophosphoric acid and enhanced chemiluminescence (ECL) reagents were obtained from Amersham (Amersham, UK). Protein A-Sepharose beads were obtained from Pharmacia (Uppsala, Sweden). Affinity purified rabbit polyclonal antibodies raised against a 16 amino acid sequence in the carboxy terminal of ERK1 (sc-93), a 14 amino acid sequence in the carboxy terminal of ERK2 (sc-154) and against a peptide corresponding to the human cyclin D1 gene product, PRAD1 (sc-92) were obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA). A rabbit polyclonal antibody (PSK-J3) raised against an epitope within the carboxy terminal domain of human CDK4 was obtained from Upstate Biotechnology Incorporated (Lake Placid, NY). BrdU labelling and detection reagents were obtained from Boehringer Mannheim (Mannheim, Germany). All other chemicals were from Sigma (St Louis, MO).

Neonatal β Cell Culture

Monolayer-forming β cells were obtained from collagenase-dispersed pancreas of neonatal rats (< 1 day-old) cultured over 4 days in medium RPMI 1640 containing 10% (v:v) fetal bovine serum (FBS) as described previously [17]. Thimerosal, which deters the rapid growth of fibroblasts was included for the first 48 h. Islet cell monolayers were established in which >90% give positive specific insulin immunofluorescence [17]. For experimentation β cell culture was continued for a further 24 h in RPMI 1640 containing 5.6 mmol/L glucose and 1.0% (v:v) FBS and cells were labelled in culture with [^{32}P]orthophosphate or [^{35}S]methionine or BrdU added as described below.

BrdU-Labelling of β Cell DNA

Collagenase-dispersed islets were seeded and cultured on glass coverslips in 100mm plates with oleoyl-LPA (1–25 μg/ml) present for the final 24 h. 5-bromo-2′deoxyuridine (10 μmol/L) was added for the final 5 h of culture. After this time cells attached to glass cover slips were ethanol-fixed, incubated with monoclonal anti-BrdU antibody solution containing nucleases to promote DNA denaturation and anti-BrdU antibody bound to single-stranded DNA was localized with fluorescein-labelled anti-mouse Ig. Cells were viewed by immunofluorescence microscopy and for each condition the percentage BrdU-positive nuclei of at least 300 cells (shown to be 92 ± 4% positive for insulin immunofluorescence in the present study) was calculated.

β cell [^{32}P] Labelling of ERK1

For whole cell ^{32}P labelling, cells were grown in 100mm plates with [^{32}P]orthophosphate (2.5 mCi/ml) in serum-depleted (1% FBS) medium for 24 h. In these experiments, when present, pertussis toxin (50 ng/ml) or mycophenolic acid (MPA, 25 μg/ml) were included in the labelling period. After this time cells were washed rapidly with three changes of warmed RPMI medium and stimulated with oleoyl-LPA (10 μg/ml) for 10 min, washed with ice-cold phosphate-buffered saline and lysed in a Triton X-100 lysis buffer, the lysates were centrifuged at 14,000 rpm for 10 min at 4°C and an aliquot of the lysate immunoprecipitated as described [18] with polyclonal antibody (sc-93), raised against p44 ERK1. ^{32}P-labelled proteins in the a pre-cleared lysate and ^{32}P-labelled immunoprecipitated proteins were resolved by 10% SDS/PAGE, transferred to nitrocellulose and exposed to X-OMAT AR film. Subsequently the filters were probed with ERK1 (sc-93) and ERK2 (sc-154) antibodies and the signal detected by ECL.

CDK4 Antibody Immunoprecipitation of [^{35}S]Methionine Biosynthetically-Labelled Proteins

Cells were grown in 100mm plates in serum-depleted medium containing [^{35}S]methionine (0.1 mCi/ml) for 24 h, when present MPA was included over this labelling time. Oleoyl-LPA (10 μg/ml) was added over the final 3 h of culture. Cells were washed as described above and lysates prepared in a 1% Nonidet P-40 lysis buffer [11]. Lysates were immunoprecipitated with affinity-purified anti-CDK4 antibody (PSK-J3) and the immunoprecipated proteins resolved as described above. After transfer filters were autoradiographed to determine ^{35}S-labelled proteins and then probed subsequently with anti-cyclin D1 antibody (SC-92) and anti-CDK4 antibody (PSK-J3), followed by ECL detection.

RESULTS

Following 24 h exposure to LPA there was a concentration-dependent increase in uptake and nuclear-incorporation of BrdU in the cells under study. The percentage β cells positive for BrdU was 24 ± 3% and 33 ± 2% for oleoyl-LPA concentrations of 10 μg/ml and 25 μg/ml, respectively, which were significantly increased over incorporation of BrdU in the basal condition (12 ± 2%, $p < 0.05$) confirming our previous observation for these cells [13].

Phosphorylated proteins of a lysate from β cells stimulated with oleoyl-LPA over 10 min are shown in Figure 1 (panel A). Several phosphoproteins showed increased labelling; specifically it was seen that in a region localized by subsequent probing with antibodies to ERK1 and ERK2, phosphoproteins were enhanced by exposure to LPA. The most apparent increase was observed in the ERK1 region and occurred in the absence of any increase in ERK1 protein (panel B). The presence of ^{32}P-labelled ERK1 protein was confirmed by immunoprecipitation using anti-ERK1 antibody (panel C). Preliminary experiments showed 10 min to be maximal for ERK1 phosphor-

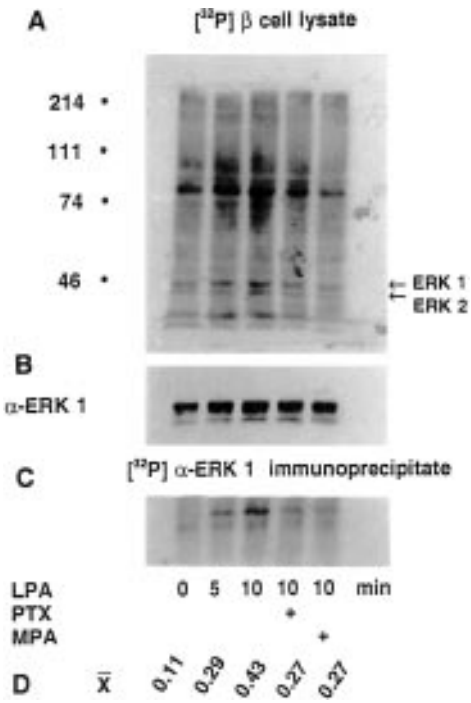

FIG. 1. β cell [^{32}P] labelling of ERK1. Panel A: Phosphoproteins in a β cell lysate are shown. The position of ERK1 and ERK2 subsequently localized by immunoblotting is indicated. Panel B: Proteins localized by subsequent immunoblotting with an anti-ERK1 antibody (α-ERK1) and ECL. Panel C: ^{32}P-labelled proteins immunoprecipitated using anti-ERK1 antibody. The results shown are from one experiment of duplicates, representative of three further experiments. Panel D: For the duplicates of the experiment shown, autoradiographs from ^{32}P α-ERK1 immunoprecipitates (Panel C) and α-ERK1 immunolocalized proteins (Panel B) were quantitated by laser densitometry and the ratios expressed as a mean (X).

ylation following LPA addition. An increase in ERK1 phosphorylation in response to LPA was not seen in cells pre-treated with pertussis toxin or with MPA (panel C).

In lysates from ^{35}S-labelled cells an antibody to CDK4 co-immunoprecipated a number of labelled cellular proteins (Figure 2, panel A), some of which remain unchanged over a 3 h incubation with LPA. At the later times of this incubation there was a modest increase in labelled proteins in the region corresponding to cyclin D1 (p33) and CDK4 (p36). This was not seen in cells pretreated with MPA (panel D). Subsequently it could be shown that levels of CDK4 protein, detected by probing with anti-CDK4 antibody (panel B) remained constant over 3 h of LPA exposure. CyclinD1 co-immunoprecipitated with CDK4 (determined by reprobing with anti-cyclin D1 antibodies, panel C) was increased only after 1 h of LPA exposure and was maximal by 2 h.

DISCUSSION

The complexes formed by CDK4 and the D-type cyclins have been described as necessary and rate limiting for progression through G1, the first gap phase of the cell cycle, but occur only after cells are committed to re-enter the cell cycle from G0 (cell cycle withdrawal). Cell cycle analysis in neonatal rat islets provides evidence that many β cells retain a limited capacity for cell division and are not maintained in an irreversible G0 phase [6]. The timing of CDK4/CyclinD1 association in the present study is in good agreement with a mid-G1 event as a 2.5 h G1 phase has been

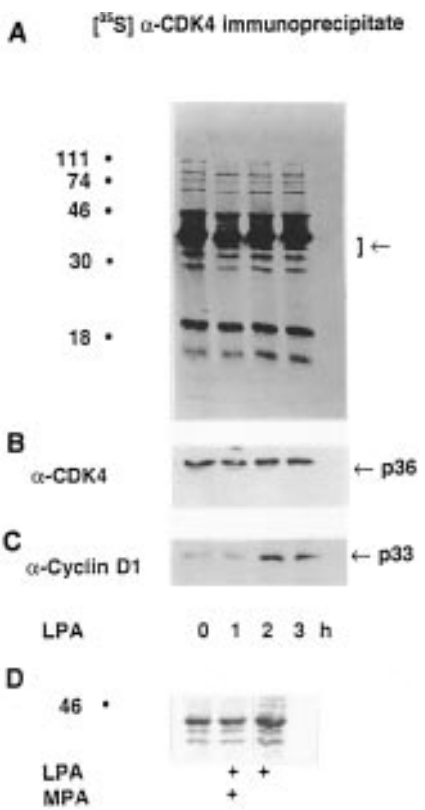

FIG. 2. CDK4 antibody immunoprecipitation of [^{35}S]methionine biosynthetically-labelled proteins. Panel A: Labelled proteins immunoprecipitated with anti-CDK4 antibody (α-CDK4). Panel B: Subsequent immunolocalization of the CDK4 protein using α-CDK4. Panel C: Immunolocalization of cyclin D in α-CDK4 immunoprecipitates using anticyclin D1 antibody (α-Cyclin D1). Panel D: Labelled proteins immunoprecipitated with anti-CDK4 antibody after 3 h exposure to LPA of cells incubated in the presence or absence of MPA. The results shown are one of duplicates from experiments representative of two further experiments.

described for cultured rat β cells [1]. In the present study, in which neonatal β cells are serum-depleted but not specifically cell cycle synchronized, it can be shown that a capacity to proliferate in response to a growth stimulus, is preceded by a rapid phosphorylation of ERK1. As seen previously for GTP association with p21ras in response to LPA in these cells [13], this phosphorylation is both guanine nucleotide-dependent (inhibited by GTP depletion following mycophenolic acid [19] and pertussis toxin sensitive. This latter aspect may reflect a G_i-protein-coupled receptor signalling following LPA [14] and/or that G_i-coupled receptor stimulation of ERK1 by LPA in rat fibroblasts is mediated by G-protein$\beta\gamma$ subunit activation of p21ras [20]. Phosphorylation of ERK2 (p42) and ERK1 (p44) has been described in adult rat pancreatic islets [21] and in an insulin-secreting beta-cell line, INS-1 [22], respectively, although this was not related to DNA synthesis. From our present and previous studies, we suggest that CDK4/cyclin D1 association is an event consequent on p21ras activation [13] and ERK1 phosphorylation as it is not seen when either event is inhibited by the reduction of guanine nucleotides. In adult mouse islets expression of the genes encoding cyclin B1, cyclin D1, p34cdc2 kinase (which may be associated with cyclin A or B partners) and p33cdk2 kinase (a cyclin D, E and A partner) has been described [23]. In those islets a low expression of cyclin B1 and p34cdc2 kinase is proposed to contribute to the low adult proliferative capacity. However, unlike CDK4/CyclinD1, this CDK/cyclin partnership contributes to the late cell cycle event in S phase and G2/M phase [9]. We describe the presence and association of two components of early G1 phase progression, which accompany a proliferative stimulus and are proposed to follow an activation of the ERK1 pathway. ERK1-related events leading to this association may provide information on the requirements and capacity of both adult and neonatal islet cells to commit to cell division.

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