Signalling pathways of insulin-like growth factor-I that are augmented by cAMP in FRTL-5 cells

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We have reported that pretreatment of rat FRTL-5 thyroid cells with thyrotropin (TSH) markedly potentiates the mitogenic response to insulin-like growth factor-I (IGF-I). The present study was undertaken to determine whether the augmentation by cAMP of IGF-I-dependent tyrosine phosphorylation of known IGF-I receptor substrates plays an important role in the cAMP-dependent potentiation of DNA synthesis induced by IGF-I. Pretreatment with TSH or dibutyryl cAMP did not affect the IGF-I-dependent tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1). In contrast, cAMP pretreatment potentiated the tyrosine phosphorylation of IRS-2 induced by IGF-I, but did not affect the amount of IRS-2. We found that the IGF-I-dependent tyrosine phosphorylation of 66 kDa Shc (Src homology collagen) was markedly increased by cAMP pretreatment, and that this change was mainly due to an increase in the levels of 66 kDa Shc protein. Under these conditions, cAMP pretreatment significantly increased binding of Grb2 (growth-factor-receptor-bound protein 2) to Shc in response to IGF-I, and activation of MAP kinase (mitogen-activated protein kinase)

induced by IGF-I was also enhanced by cAMP. The presence of PD98059, an inhibitor of MEK (MAP-kinase/Erk kinase), during treatment with IGF-I partially inhibited the cAMP-dependent augmentation of DNA synthesis in response to IGF-I. On the other hand, cAMP pretreatment increased binding of the phosphoinositide 3-kinase (PI 3-kinase) p85 subunit to IRS-2, which was reflected in PI 3-kinase activity. LY294002, a PI 3-kinase inhibitor, strongly depressed IGF-I-dependent DNA synthesis after pretreatment with and without TSH or dibutyryl cAMP. Our results suggest that the interaction between cAMP-dependent and IGF-I-dependent pathways leads to an augmentation of cell proliferation, which is mediated, at least in part, through the MAP kinase and PI 3-kinase signalling pathways. These effects are mediated by changes in tyrosine phosphorylation of IGF-I receptor substrates, including IRS-2 and Shc.

Key words: IGF-I, IRS-1, IRS-2, Shc.

INTRODUCTION

Insulin-like growth factor-I (IGF-I) can stimulate both cell proliferation and differentiation in many cell types [1,2]. IGF-I by itself often has weak biological effects, which are greatly potentiated in the presence of other hormones or growth factors [3–10]. Although cAMP has been reported to antagonize tyrosine kinase receptor-activated pathways in some cells [11–19], we have shown previously that pretreatment of FRTL-5 cells with thyrotropin (TSH), which acts through a cAMP-dependent pathway, potentiates DNA synthesis induced by IGF-I [20,21]. The present studies are part of ongoing attempts to elucidate the signalling pathways through which such synergistic actions are mediated.

Pretreatment of FRTL-5 cells with TSH or other substances that increase cAMP levels enhances the IGF-I-dependent tyrosine phosphorylation of several intracellular proteins, predominantly those with molecular masses of 175–185 kDa or 90–100 kDa [21]. Under these conditions, we reported that cAMP did not increase IGF-I receptor number or kinase activity [22]. These results suggested that the interaction between cAMP-dependent and IGF-I-dependent pathways leads to an augmentation of cell proliferation through changes in IGF-I-dependent tyrosine

phosphorylation at a step distal to IGF-I receptor kinase activation.

Binding of IGF-I to its specific receptor on a target cell membrane is followed by activation of tyrosine kinase in the β -subunit of the IGF-I receptor [22]. The activated IGF-I receptor in turn phosphorylates specific substrates, in particular insulin receptor substrate-1 (IRS-1), IRS-2 and Src homology collagen (Shc), as well as putative substrates such as Crk (CT10 regulator of kinase) and Jaks (Janus-activated kinases) [23–28]. Recently it has become clear that phosphorylated tyrosine residues of IRS-1, IRS-2 and Shc are recognized by various signalling molecules that contain a Src homology 2 (SH2) domain [29,30]. These include Grb2 (growth-factor-receptor-bound protein 2) and the 85 kDa regulatory subunit of phosphoinositide (phosphatidylinositol) 3-kinase (PI 3-kinase), suggesting that binding of tyrosine-phosphorylated substrates to these SH2-domain-containing proteins mediates IGF-I bioactivities. These associations are thought to stimulate the mitogen-activated protein kinase (MAP kinase) cascade or the PI 3-kinase cascade, well known signalling pathways that mediate many significant actions of growth factors [31].

The present study was undertaken to determine whether the augmentation by cAMP of IGF-I-dependent tyrosine

Abbreviations used: CH2 domain, collagen-homology 2 domain; Erk, extracellular-signal-regulated kinase; Grb2, growth-factor-receptor-bound protein 2; HBSS, Hanks balanced salt solution; IGF-I, insulin-like growth factor-I; IRS, insulin receptor substrate; MAP kinase, mitogen-activated protein kinase; MEK, MAP-kinase/Erk kinase; NBS, newborn bovine serum; PI 3-kinase, phosphoinositide (phosphatidylinositol) 3-kinase; SH, Src homology; Shc, Src homology collagen; TSH, thyrotropin.

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phosphorylation is specifically reflected in increased phosphorylation of the known IGF-I receptor substrates IRS-1, IRS-2 and Shc. In addition, we investigated whether potentiation of tyrosine phosphorylation causes an increase in binding of these substrates to Grb2 or to the 85 kDa regulatory subunit of PI 3-kinase, followed by activation of MAP kinase or PI 3-kinase.

EXPERIMENTAL

Materials

Coon's modified Ham's F-12 medium (Coon's F-12) was purchased from Sigma (St. Louis, MO, U.S.A.); newborn bovine serum (NBS) was from Nichirei (Tokyo, Japan); Hanks balanced salt solution (HBSS) was from Nissui (Tokyo, Japan); bovine insulin, bovine TSH and human transferrin were purchased from Sigma; penicillin was obtained from Ban-yu Pharmaceutical Co. (Tokyo, Japan), streptomycin and kanamycin were from Meiji Seika Co. (Tokyo, Japan); and amphotericin B (fungizone) was from Sankyo Pharmaceutical Co. (Tokyo, Japan). Purified bovine TSH (30 units/mg) for biological studies was generously donated by the National Hormone and Pituitary Program [National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), Bethesda, MD, U.S.A.], and recombinant human IGF-I was a gift from Dr Toshiaki Ohkuma (Fujisawa Pharmaceutical Co., Osaka, Japan). Leupeptin and pepstatin were kindly donated by Dr Takaaki Aoyagi (Institute of Microbial Chemistry, Tokyo, Japan). Anti-phosphotyrosine monoclonal antibody was kindly provided by Dr Takao Yamori (Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan). Anti-(rat IRS-1) antibody for immunoprecipitation, anti-(human Grb2) antibody (against N-terminal SH3 region), anti-p85 antibody (against N-terminal SH2 region) and anti-Shc antibody were purchased from Upstate Biotechnology Inc. (Lake Placid, NY, U.S.A.). Anti-(human IRS-1) antibody for immunoblotting and anti-(rat IRS-2) (M-19) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). Anti-phosphoErk antibody (where Erk is extracellular-signal-regulated kinase) was from Transduction Laboratories (Lexington, KY, U.S.A.). Anti-IRS-3 and anti-IRS-4 antibodies were kindly donated by Dr Tomoichiro Asano (Faculty of Medicine, The University of Tokyo, Tokyo, Japan). [γ - 32 P]ATP (> 6000 Ci / mmol) was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Other chemicals were of reagent grade available commercially.

Cell cultures

FRTL-5 cells (A.T.C.C. no. CRL8305), a line of rat thyroid follicular cells developed by Ambesi-Impionbato et al. [31a], were generously provided by Dr Leonard Kohn (Section of Cell Regulation, NIDDK). Cells were routinely cultured in Coon's F-12 medium including three hormones (3H): TSH (1 m-unit/ml), insulin (10 μ g/ml) and transferrin (5 μ g/ml). Cells were cultured in 150-cm² flasks (IWAKI, Tokyo, Japan) at 37 °C in an atmosphere of 95% air/5% CO₂ in a humidified incubator [20]. All media contained penicillin (100 units/ml), streptomycin (10 μ g/ml), kanamycin (1 μ g/ml) and amphotericin B (0.2 μ g/ml).

Cell treatments

FRTL-5 cells (1×10^6 cells/8 ml) were sparsely seeded in 100-mm dishes (IWAKI) in Coon's F-12 medium containing 5% (v/v) NBS and 3H. For DNA synthesis assays, cells (1×10^4 cells/500 μ l per well) were seeded in 48-well plates (IWAKI). After 6 days,

the cells were washed twice with HBSS, and the cultures were continued for an additional 24 h in Coon's F-12 medium containing 0.1% (w/v) BSA without NBS and 3H until cells became quiescent. The medium was then replaced with the experimental medium (Coon's F-12 medium containing 0.1% BSA) without or with TSH (1 nM) or dibutyryl cAMP (1 mM), and the cells were pretreated for 24 h. At the end of pretreatment, the cells were washed twice with HBSS. The cells were then treated with IGF-I (100 ng/ml) for periods of time specified for each experiment.

Immunoprecipitation of IRSs or Shc, followed by immunoblotting with anti-phosphotyrosine antibody or antibody against each protein

As described above, the quiescent FRTL-5 cells were pretreated without or with TSH (1 nM) or dibutyryl cAMP (1 mM) for 24 h, followed by treatment with IGF-I (100 ng/ml) for various times. The cells were then harvested at 0 °C in 400 μ l of immunoprecipitation buffer containing 50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM Na₃VO₄, 10 mM EDTA, 1 mM EGTA, 1.5 mM MgCl₂, 1% Triton X-100, 100 kallikrein-inactivating units/ml aprotinin, 20 μ g/ml PMSF, 10 μ g/ml leupeptin, 5 μ g/ml pepstatin and 10 mg/ml *p*-nitrophenyl phosphate. The lysates were centrifuged at 12000 *g* for 10 min at 4 °C. The supernatant was diluted with immunoprecipitation buffer to 1 mg of protein/ml as a final concentration. The protein assay was carried out using a protein assay kit (Bio-Rad, Hercules, CA, U.S.A.). The lysates were incubated with anti-IRS-1 antibody (10 μ g), anti-IRS-2 antibody (10 μ g), anti-Shc antibody (10 μ g), anti-IRS-3 antibody (10 μ l) or anti-IRS-4 antibody (10 μ l) for 12 h at 4 °C; 40 μ l of Protein A-Sepharose [50% (v/v); Pharmacia Biotech] was then added and the incubation was continued for 2 h. The immunoprecipitates were collected by centrifugation at 4 °C, washed three times with immunoprecipitation buffer containing 300 mM NaCl, and boiled for 5 min in a mixture of 60 μ l of immunoprecipitation buffer and 30 μ l of 3 $\times$ Laemmli sample buffer (9% SDS, 15% glycerol, 30 mM Tris/HCl, pH 7.8, 0.05% Bromophenol Blue and 6% 2-mercaptoethanol). These samples were then stored at -80 °C until electrophoresis.

Portions of each sample (containing equal amounts of protein) were run on SDS/PAGE followed by immunoblotting analysis, as described previously [32]. The transferred membrane was incubated for 2 h at room temperature in blocking buffer containing a monoclonal anti-phosphotyrosine antibody (1:1000 dilution) or anti-Grb2 antibody (1:1000), or incubated overnight at room temperature in the blocking buffer containing anti-IRS-1 antibody (1:200), anti-IRS-2 antibody (1:500), anti-Shc antibody (1:1000) or anti-p85 antibody (1:2000). The results were quantified using the NIH Image computer program (Version 1.61).

Immunoblotting of cell lysates using anti-IRS-1, anti-IRS-2 and anti-Shc antibodies

As described above, quiescent FRTL-5 cells were treated without or with TSH (1 nM) or dibutyryl cAMP (1 mM) for 24 h, and then harvested at 0 °C in 400 μ l of immunoprecipitation buffer. The lysates were centrifuged at 12000 *g* for 10 min at 4 °C. The protein assay of the supernatants was carried out using a protein assay kit (Bio-Rad), and the protein content of the lysates was adjusted to 1 mg/ml by addition of immunoprecipitation buffer. A portion of 200 μ l of concentrated 3 $\times$ Laemmli sample buffer containing 9% SDS and 6% 2-mercaptoethanol was added to 400 μ l of supernatant, and the mixture was boiled for 5 min.

These samples were stored at -80°C until electrophoresis. Portions of each sample containing the same amount of protein were run on SDS/PAGE, followed by immunoblotting using anti-IRS-1 antibody, anti-IRS-2 antibody or anti-Shc antibody.

MAP kinase assay by immunoblotting with anti-phosphoErk antibody

As described above, quiescent FRTL-5 cells were pretreated without or with TSH (1 nM) or dibutyryl cAMP (1 mM) for 24 h, followed by treatment with IGF-I (100 ng/ml) for 5 min. Cells were lysed and homogenized at 4°C in 400 μl of buffer containing 80 mM β -glycerophosphate, pH 7.4, 15 mM MgCl_2 , 20 mM EGTA, 1 mM Na_3VO_4 , 20 $\mu\text{g}/\text{ml}$ PMSF, 10 $\mu\text{g}/\text{ml}$ leupeptin, 5 $\mu\text{g}/\text{ml}$ pepstatin and 1 mM benzamidine. The lysates were centrifuged at 12000 g for 15 min at 4°C . The supernatants were recovered and the concentration of protein was measured using a protein assay kit. Portions of each sample containing the same amount of protein were run on SDS/12.5%-PAGE and immunoblotting was performed as described above, but using anti-phosphoErk polyclonal antibody (1:1000). The amounts of phosphorylated Erk1 (lower mobility) and Erk2 (higher mobility) were taken to represent activated Erk1 and Erk2 respectively.

PI 3-kinase activity assay

As described above, quiescent FRTL-5 cells were pretreated without or with TSH (1 nM) or dibutyryl cAMP (1 mM) for 24 h, followed by treatment with IGF-I (100 ng/ml) for 2 min. Cells were lysed at 4°C in 300 μl of Nonidet P-40 buffer (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.5 mM Na_3VO_4 , 1 mM EDTA, 1% Nonidet P-40, 100 kallikrein-inactivating units/ml aprotinin, 20 $\mu\text{g}/\text{ml}$ PMSF, 10 $\mu\text{g}/\text{ml}$ leupeptin, 5 $\mu\text{g}/\text{ml}$ pepstatin and 10 mg/ml *p*-nitrophenyl phosphate). The lysates were centrifuged at 12000 g for 10 min at 4°C . The supernatant was incubated with anti-IRS-1 antibody (10 μg), anti-IRS-2 antibody (10 μg) or anti-phosphotyrosine antibody (20 μl) for 12 h at 4°C ; 10 μl of Protein A-Sepharose (50%, v/v) was then added and the incubation was continued for 2 h. The immunoprecipitates were collected by centrifugation at 4°C , washed once each with Nonidet P-40 buffer, LiCl buffer (100 mM Tris/HCl, pH 7.5, and 500 mM LiCl), distilled water and TNE buffer (10 mM Tris/HCl, pH 7.5, 150 mM NaCl and 1 mM EDTA), and finally resuspended in 45 μl of reaction buffer (20 mM Tris/HCl, pH 7.5, 100 mM NaCl and 0.5 mM EGTA).

The PI 3-kinase assay was carried out using the method of Whitman et al. [33], with slight modifications. Briefly, the PI 3-kinase assay was initiated by incubation of immunocomplex in 45 μl of reaction buffer with 5 μl of $[\gamma\text{-}^{32}\text{P}]\text{ATP}/\text{MgCl}_2/\text{phosphatidylinositol}$ to give final concentrations of 20 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (4 $\mu\text{Ci}/\text{mmol}$), 20 mM MgCl_2 and 20 μg of phosphatidylinositol (Avanti, Pharmacia Biotech) at 25°C for 30 min. After incubation, 100 μl of chloroform/methanol/HCl (10:20:1, by vol.) was added to the reaction mixture to stop the reaction. A lipid product was extracted, spotted on to a silica gel plate and developed with chloroform/methanol/ $\text{NH}_4\text{OH}/\text{water}$ (43:38:6:6, by vol.). ^{32}P radioactivity incorporated into phosphatidylinositol was measured by autoradiography as PI 3-kinase activity. Each experimental point represents the mean from three replicate dishes.

DNA synthesis assay

Determination of [*methyl*- ^3H]thymidine incorporation into DNA was performed as described previously [20]. In all experiments,

each experimental point represents the mean from three replicate wells.

RESULTS

Effects of cAMP pretreatment on tyrosine phosphorylation of IRSs stimulated by IGF-I

To test the effects of pretreatment with TSH or dibutyryl cAMP (cAMP pretreatment) on tyrosine phosphorylation of IGF-I receptor substrates, such as IRS-1 and IRS-2, FRTL-5 cells were preincubated without or with TSH or dibutyryl cAMP for 24 h. We then analysed the tyrosine phosphorylation of each substrate by immunoprecipitation using an antibody against each protein, followed by immunoblotting with an anti-phosphotyrosine antibody. The tyrosine phosphorylation of IRS-1 increased after IGF-I treatment, and this increase was not affected by cAMP pretreatment (Figures 1A and 1B). In the case of IRS-2, tyrosine phosphorylation in response to IGF-I was clearly potentiated by pretreatment with cAMP (Figures 2A and 2B). However, under

Figure 1 Effects of TSH and dibutyryl cAMP on tyrosine phosphorylation of IRS-1 stimulated by IGF-I

(A) Quiescent FRTL-5 cells were pretreated without (No additives) or with TSH (1 nM) for 24 h, washed twice with HBSS and incubated with 100 ng/ml IGF-I for various time (0, 1, 2, 5 and 10 min). After treatment, cells were harvested and the cell lysate was subjected to immunoprecipitation (IP) with anti-IRS-1 antibody, followed by SDS/8%-PAGE and immunoblotting (IB) with anti-phosphotyrosine antibody (αPY), as described in the Experimental section. These experiments were performed on three independent occasions, and a representative blot is presented. (B) Quiescent FRTL-5 cells were pretreated without (No) or with TSH (1 nM) or dibutyryl cAMP (cAMP; 1 mM) for 24 h, washed twice with HBSS and incubated without or with 100 ng/ml IGF-I for 2 min. After treatment, cells were harvested and the cell lysate was subjected to immunoprecipitation with anti-IRS-1 antibody, followed by SDS/8%-PAGE and immunoblotting with anti-phosphotyrosine antibody and anti-IRS-1 antibody, as described in the Experimental section. IRS-1 tyrosine phosphorylation and IRS-1 protein content were quantified by NIH Image software. IRS-1 tyrosine phosphorylation corrected for its protein content is expressed as a percentage of IGF-I-dependent tyrosine phosphorylation in cells pretreated with no additives, and results represent means $\pm$ S.E.M. of three independent experiments. (C) Quiescent FRTL-5 cells were treated without (No) or with TSH (1 nM) or dibutyryl cAMP (cAMP; 1 mM) for 24 h. Cells were harvested without exposure to IGF-I, and the cell lysate was subjected to immunoblotting with anti-IRS-1 antibody as described in the Experimental section. The amount of IRS-1 in cells treated with no additives, TSH or cAMP was quantified by NIH Image software. The amount of IRS-1 is expressed as a percentage of the IRS-1 content in cells treated with no additives, and the results represent means $\pm$ S.E.M. of three independent experiments.

Figure 2 Effects of TSH and dibutyl cAMP on tyrosine phosphorylation of IRS-2 stimulated by IGF-I

(A) Quiescent FRTL-5 cells were pretreated without (No additives) or with TSH (1 nM) for 24 h, washed twice with HBSS and incubated with 100 ng/ml IGF-I for various times (0, 1, 2, 5 and 10 min). After treatment, cells were harvested and the cell lysate was subjected to immunoprecipitation (IP) with anti-IRS-2 antibody, followed by SDS/8%-PAGE and immunoblotting (IB) with anti-phosphotyrosine antibody (α PY), as described in the Experimental section. These experiments were performed on three independent occasions, and a representative blot is presented. (B) Quiescent FRTL-5 cells were pretreated without (No) or with TSH (1 nM) or dibutyl cAMP (cAMP; 1 mM) for 24 h, washed twice with HBSS and incubated without or with 100 ng/ml IGF-I for 2 min. After treatment, cells were harvested and the cell lysate was subjected to immunoprecipitation with anti-IRS-2 antibody, followed by SDS/8%-PAGE and immunoblotting with anti-phosphotyrosine antibody and anti-IRS-2 antibody, as described in the Experimental section. The quantitative analysis was performed as described for Figure 1(B); * indicates a significant difference ($P < 0.05$) between values obtained in cells pretreated with no additives and those pretreated with TSH or cAMP, followed by IGF-I treatment in all cases (unpaired *t*-test). (C) Quiescent FRTL-5 cells were treated without (No) or with TSH (1 nM) or dibutyl cAMP (cAMP; 1 mM) for 24 h. Cells were harvested without exposure to IGF-I and the cell lysate was subjected to immunoblotting with anti-IRS-2 antibody, as described in the Experimental section. These experiments were performed on three independent occasions. Densitometric analysis of the blot was performed as described for Figure 1(C).

these conditions cAMP did not affect the amounts of these proteins, as assessed by analysing the lysates by immunoblotting with antibodies against IRS-1 and IRS-2 (Figures 1C and 2C). We could not detect tyrosine phosphorylation of IRS-3 and IRS-4 in this cell line (results not shown).

Effects of cAMP pretreatment on tyrosine phosphorylation of Shc stimulated by IGF-I

We next studied the tyrosine phosphorylation of Shc in response to IGF-I. In FRTL-5 cells we observed the tyrosine phosphorylation of three isoforms of Shc, with molecular masses of 46 kDa, 52 kDa and 66 kDa, and an IGF-I-dependent increase in tyrosine phosphorylation was observed in each case (Figure 3A). Of these three proteins, cAMP pretreatment significantly potentiated the tyrosine phosphorylation of 66 kDa Shc induced by IGF-I (Figure 3B). In Figure 3(C) the degree of tyrosine phosphorylation of 66 kDa Shc induced by IGF-I is related to the amount of 66 kDa Shc protein in the immunoprecipitates. The ratio was not significantly changed by cAMP pretreatment, suggesting that an increase in the phosphorylation of 66 kDa Shc

Figure 3 Effects of TSH and dibutyl cAMP on tyrosine phosphorylation of Shc stimulated by IGF-I

(A)–(C) Quiescent FRTL-5 cells were pretreated without (No) or with TSH (1 nM) or dibutyl cAMP (cAMP; 1 mM) for 24 h, washed twice with HBSS and incubated without or with 100 ng/ml IGF-I for 5 min. After treatment, cells were harvested and the cell lysate was subjected to immunoprecipitation (IP) with anti-Shc antibody, followed by SDS/10%-PAGE and immunoblotting (IB) with anti-phosphotyrosine antibody or anti-Shc antibody, as described in the Experimental section. These experiments were performed on three independent occasions, and a representative blot is shown (A). The quantitative analysis of tyrosine phosphorylation of 66 kDa Shc (p66 Shc) (B) was performed as described for Figure 1(B); * indicates a significant difference ($P < 0.05$) between values obtained in cells pretreated with no additives and those pretreated with TSH or cAMP, followed by IGF-I treatment in all cases (unpaired *t*-test). Panel (C) shows the degree of tyrosine phosphorylation of p66 Shc corrected for the amount of protein. (D)–(E) Quiescent FRTL-5 cells were treated without (No) or with TSH (1 nM) or dibutyl cAMP (cAMP; 1 mM) for 24 h. Cells were harvested without exposure to IGF-I and the cell lysate was subjected to immunoblotting (IB) with anti-Shc antibody, as described in the Experimental section. These experiments were performed on three independent occasions and a representative blot is shown (D). The densitometric analysis of the amount of 66 kDa Shc (E) was performed as described for Figure 1(C); * indicates a significant difference ($P < 0.05$) between values obtained in cells treated with no additives and those treated with TSH or cAMP (unpaired *t*-test).

was mainly due to an increase in its amount. Figures 3(D) and 3(E) also show that cAMP treatment caused a marked increase in the amount of 66 kDa Shc.

Effects of cAMP pretreatment on the association of tyrosine-phosphorylated IRS-1, IRS-2 or Shc with Grb2, followed by activation of MAP kinase

We then examined whether TSH or dibutyl cAMP pretreatment affected binding of tyrosine-phosphorylated substrates to Grb2, using co-immunoprecipitation with an antibody against IRS-1, IRS-2 or Shc. Association of Grb2 with IRS-2 or Shc was

Figure 4 Effects of pretreatment with TSH or dibutyryl cAMP on binding of Grb2 to tyrosine-phosphorylated substrates stimulated by IGF-I

Quiescent FRTL-5 cells were pretreated without (No) or with TSH (1 nM) or dibutyryl cAMP (cAMP; 1 mM) for 24 h, washed twice with HBSS and incubated without or with 100 ng/ml IGF-I, for 2 min in the case of IRS-2 and for 5 min in the case of Shc. After treatment, cells were harvested and the cell lysate was subjected to immunoprecipitation with anti-IRS-2 antibody (A) or anti-Shc antibody (B and C), followed by SDS/12.5%-PAGE and immunoblotting with anti-Grb2 antibody plus anti-IRS-2 antibody or anti-Shc antibody, as described in the Experimental section. These experiments were performed on three independent occasions. The amounts of Grb2 and IRS-2 or Shc were quantified using NIH Image software. The extent of interaction between Grb2 and IRS-2 corrected for the amount of IRS-2 (A), the amount of Grb2 bound to Shc (B), and the extent of the interaction between Grb2 and Shc corrected for the amount of 66 kDa Shc (p66 Shc) (C) are presented as described for Figure 1(B); * indicates a significant difference ($P < 0.05$) between values obtained in cells pretreated with no additives and those pretreated with TSH or cAMP, followed by IGF-I treatment in all cases (unpaired *t*-test).

Figure 5 Effects of TSH or dibutyryl cAMP pretreatment on Erk activity stimulated by IGF-I

Quiescent FRTL-5 cells were pretreated without (No) or with TSH (1 nM) or dibutyryl cAMP (cAMP; 1 mM) for 24 h, washed twice with HBSS and incubated without or with 100 ng/ml IGF-I for 5 min. After treatment, cells were harvested and the cell lysate was subjected to immunoblotting with anti-phosphoErk antibody, as described in the Experimental section. These experiments were performed on three independent occasions. The amounts of phosphoErk1 (p44 MAP kinase) (A) and phosphoErk2 (p42 MAP kinase) (B) were quantified using NIH Image software, and are expressed as a percentage of the respective phosphoErk in cells pretreated with no additives followed by IGF-I treatment. Results represent the means $\pm$ S.E.M. of three independent experiments; * indicates a significant difference ($P < 0.05$) between values obtained in cells pretreated with no additives and those pretreated with TSH or cAMP, followed by IGF-I treatment in all cases (unpaired *t*-test).

detected in response to IGF-I (Figures 4A and 4B), but association of Grb2 with IRS-1 was not observed (results not shown). cAMP pretreatment markedly increased Grb2 binding to Shc and slightly increased its binding to IRS-2 (Figures 4A and 4B). Figure 4(C) shows the extent of the interaction between Grb2 and Shc corrected for the amount of 66 kDa Shc protein, which was not affected by cAMP pretreatment.

From the results of the phosphoErk assay, cAMP increased the IGF-I-dependent phosphorylation of Erk, indicating that activation of Erk in response to IGF-I was enhanced by cAMP pretreatment (Figure 5). cAMP pretreatment was more effective in potentiating IGF-I-dependent Erk2 activation compared with that of Erk1.

Figure 6 Effects of TSH or dibutyryl cAMP pretreatment on binding of the p85 regulatory subunit of PI 3-kinase to tyrosine-phosphorylated substrates stimulated by IGF-I

Quiescent FRTL-5 cells were pretreated without (No) or with TSH (1 nM) or dibutyryl cAMP (cAMP; 1 mM) for 24 h, washed twice with HBSS and incubated without or with 100 ng/ml IGF-I for 2 min. After treatment, cells were harvested and the cell lysate was subjected to immunoprecipitation with anti-IRS-1 antibody (A) or anti-IRS-2 antibody (B), followed by SDS/8%-PAGE and immunoblotting with anti-(p85 subunit of PI 3-kinase) antibody and an antibody against the respective IRS, as described in the Experimental section. These experiments were performed on three independent occasions. The amounts of p85 PI 3-kinase and of each IRS were quantified using NIH Image software. The extent of the interaction between the p85 subunit and IRS-1 or IRS-2, corrected for the amount of IRS, is presented as described for Figure 1(B); * indicates a significant difference ($P < 0.05$) between values obtained in cells pretreated with no additives and those pretreated with TSH or cAMP, followed by IGF-I treatment in all cases (unpaired *t*-test).

Effects of cAMP pretreatment on the association of tyrosine-phosphorylated IRS-1 or IRS-2 with the p85 subunit of PI 3-kinase, followed by activation of PI 3-kinase

We also examined whether TSH or dibutyryl cAMP pretreatment affected the binding of tyrosine-phosphorylated substrates to the p85 regulatory subunit of PI 3-kinase, using co-immunoprecipitation with an antibody against IRS-1 or IRS-2. The PI 3-kinase p85 subunit was associated with tyrosine-phosphorylated IRS-1 and IRS-2 in response to IGF-I, but basal binding of p85 to IRS-1 or IRS-2 was not observed (Figures 6A and 6B). Although cAMP pretreatment decreased PI 3-kinase p85 subunit binding to IRS-1, its association with IRS-2 was increased by

Figure 7 Effects of TSH or dibutyryl cAMP pretreatment on PI 3-kinase activity stimulated by IGF-I

Quiescent FRTL-5 cells were pretreated without (No) or with TSH (1 nM) or dibutyryl cAMP (cAMP; 1 mM) for 24 h, washed twice with HBSS and incubated with 100 ng/ml IGF-I for 2 min. PI 3-kinase activity in immunocomplexes obtained using anti-IRS-1 antibody (A), anti-IRS-2 antibody (B) or anti-phosphotyrosine antibody (C) was measured as described in the Experimental section. Each treatment was performed in triplicate in three independent experiments. PI 3-kinase activity is expressed as a percentage of the activity in cells pretreated with no additives followed by IGF-I treatment. Results represent means $\pm$ S.E.M. of three independent experiments; * indicates a significant difference ($P < 0.05$) between IGF-I-dependent activities in cells pretreated with no additives and those treated with TSH or cAMP (unpaired *t*-test).

Figure 8 Effects of a MEK inhibitor or a PI 3-kinase inhibitor on DNA synthesis induced by cAMP and IGF-I

Quiescent FRTL-5 cells were pretreated with no additives (No) or with TSH (1 nM) or dibutyryl cAMP (cAMP; 1 mM) for 24 h. The cells were then washed three times with HBSS and incubated with or without IGF-I (100 ng/ml) for an additional 24 h in the absence or presence of various concentrations of PD98059 (A) or LY294002 (B). [^3H]Thymidine incorporation into DNA was measured during the final 4 h, as described in the Experimental section. Results are means $\pm$ S.E.M. of triplicate wells; * indicates a significant difference ($P < 0.05$) in thymidine incorporation in the presence of an inhibitor compared with in the absence of an inhibitor under each treatment condition.

cAMP pretreatment. Under these conditions, IGF-I-induced PI 3-kinase activity bound to IRS-2, but not that bound to IRS-1, was enhanced by cAMP pretreatment (Figures 7A and 7B). In addition, IGF-I caused PI 3-kinase activation in the immunoprecipitates, as assessed using an anti-phosphotyrosine antibody, and IGF-I-dependent PI 3-kinase activation was significantly potentiated by cAMP pretreatment (Figure 7C).

Effects of inhibition of MAP-kinase/Erk kinase (MEK) or PI 3-kinase on DNA synthesis in cells treated with cAMP and IGF-I

To study the physiological roles of the cAMP-dependent potentiation of Erk and PI 3-kinase activities stimulated by IGF-I, we measured DNA synthesis in cells pretreated with TSH or dibutyryl cAMP for 24 h, followed by IGF-I treatment for 24 h in the presence or absence of the MEK inhibitor PD98059 or the PI 3-kinase inhibitor LY294002. PD98059 partially inhibited a cAMP-dependent increase in DNA synthesis induced by IGF-I, in a concentration-dependent manner (Figure 8A). LY294002

strongly depressed IGF-I-dependent DNA synthesis after pretreatment with or without TSH or dibutyryl cAMP (Figure 8B).

DISCUSSION

We showed previously that pretreatment of FRTL-5 cells with cAMP potentiates the IGF-I-dependent tyrosine phosphorylation of various intracellular proteins, such as those of molecular mass 175–185 kDa and 90–100 kDa [21], although these proteins were not identified. These results suggested that the synergism between cAMP and IGF-I in the growth of these cells was correlated with changes in IGF-I-dependent tyrosine phosphorylation. Activation of cAMP-dependent signalling pathways has been reported to depress growth-factor-dependent signals [11–19], which are typically mediated by activation of tyrosine kinase receptors; however, especially in endocrine cells, it has become clear that cAMP may enhance IGF-I-dependent signals through novel mechanisms [34–37]. Accordingly, in the present study we investigated whether cAMP increases the tyrosine

phosphorylation of known substrates of the IGF-I receptor, followed by enhancement of IGF-I-dependent signals, in FRTL-5 cells.

In FRTL-5 cells, brief treatment with cAMP has been shown to enhance insulin receptor or IGF-I receptor activation in response to the appropriate ligand; however, this enhancement vanished after a 2 h incubation with TSH [38]. In contrast, we have reported that prolonged cAMP treatment, e.g. for 24 h, decreased the IGF-I receptor number, but did not affect its affinity [21]. Our previous experiments further demonstrated that cAMP did not affect IGF-I-dependent autophosphorylation of the IGF-I receptor, or activation of its tyrosine kinase in response to IGF-I [21]. These results indicated that the long-term effects of cAMP on changes in tyrosine phosphorylation in response to IGF-I occurred at a step distal to tyrosine kinase activation of the IGF-I receptor.

In the present study, we first showed that tyrosine phosphorylation of IRS-2 stimulated by IGF-I is enhanced by cAMP pretreatment, but the IGF-I-dependent tyrosine phosphorylation IRS-1 is not (Figures 1 and 2). This enhancement requires long-term stimulation of the cAMP-dependent signalling pathway (results not shown). These results clearly indicate that tyrosine phosphorylation of each IRS is regulated in a distinct manner, even though IRS family members possess a similar structure [39], suggesting that each IRS has a different physiological role. Based on the results of immunoprecipitation with an antibody against each protein, tyrosine-phosphorylated proteins in the 175–185 kDa range include IRS-1 and IRS-2, but not IRS-4. During cAMP treatment, the amounts of IRS-1 and IRS-2 proteins, as judged by immunoblot analysis, were not changed significantly (Figures 1C and 2C), suggesting that the increase in the tyrosine phosphorylation of IRS-2 is not due to an increase in the amount of protein. It is possible to speculate that cAMP treatment modulates IRS-2 to increase the affinity of an IGF-I receptor or to decrease the affinity of a protein-tyrosine phosphatase to dephosphorylate IRS-2, depresses protein-tyrosine phosphatase activities, or changes the localization of IRS-2. Work to elucidate the mechanisms of the cAMP-dependent potentiation of tyrosine phosphorylation of IRS-2 induced by IGF-I is in progress in our laboratory.

Shc has been shown to have three protein isoforms, with molecular masses of 46 kDa, 52 kDa and 66 kDa. These isoforms are synthesized by using different translation start sites from two mRNAs produced by alternative splicing: one encodes 46 kDa and 52 kDa Shc, with the other encoding 46 kDa, 52 kDa and 66 kDa Shc [40,41]. In FRTL-5 cells, low-level tyrosine phosphorylation of 46 kDa and 52 kDa Shc was detected without IGF-I treatment. In contrast, IGF-I stimulated the tyrosine phosphorylation of 46 kDa, 52 kDa and 66 kDa Shc, and cAMP pretreatment prominently potentiated the IGF-I-dependent tyrosine phosphorylation of 66 kDa Shc (Figures 3A and 3B). In addition, cAMP treatment increased the amount of 66 kDa Shc protein (Figures 3D and 3E), and in fact the degree of IGF-I-dependent tyrosine phosphorylation of 66 kDa Shc normalized to its amount in the immunoprecipitates was not changed by cAMP pretreatment (Figure 3C), suggesting that cAMP-dependent potentiation of tyrosine phosphorylation of 66 kDa Shc induced by IGF-I is mainly due to an increase in the amount of protein. This mechanism could be an important point of interaction between the signalling pathways of cAMP and IGF-I. The expression and tyrosine phosphorylation of these three Shc isoforms in response to growth factors has been reported to be regulated differently in various cell types [41,42]. Based on these results, each isoform may have a different role in controlling cell behaviour. It has been reported that, unlike the other two

isoforms, 66 kDa Shc has a collagen-homology 2 domain (CH2 domain) [41]. Studies of the physiological roles of CH2 domains are clearly required in the light of our findings and the intriguing findings of Pelicci and co-workers [41] that the CH2 domain is a negative regulator of the *c-fos* promoter.

It is well known that tyrosine-phosphorylated IRS family proteins and Shc are recognized by Grb2, one of the family of adaptor signal molecules that have SH2 and SH3 domains [43]. Grb2 associates with Sos, which is a GDP/GTP exchanger of Ras. These associations are followed by activation of the MAP kinase cascade, which is an important signal transduction pathway for cell growth and differentiation [44,45]. In FRTL-5 cells, we demonstrated that the amount of Shc–Grb2 complex formed in response to IGF-I was significantly increased by cAMP pretreatment (Figure 4B). However, the extent of the interaction between Shc and Grb2, when corrected for the amount of 66 kDa Shc protein in the immunoprecipitates, was not affected by cAMP pretreatment (Figure 4C). This suggests that the cAMP-dependent increase in binding of Grb2 to Shc induced by IGF-I was due mainly to an increase in the amount of 66 kDa Shc protein in response to cAMP treatment, as was also seen for the cAMP-dependent potentiation of 66 kDa Shc tyrosine phosphorylation induced by IGF-I. On the other hand, Grb2 binding to phosphorylated IRS-2 in response to IGF-I was slightly enhanced by cAMP pretreatment (Figure 4A), and this enhancement effect of cAMP was much smaller than its effect on Grb2 binding to Shc (Figure 4B). Since binding of Grb2 to phosphorylated IRS-1 was not detected in the present study, we assumed that the amount of Grb2 binding to IRS-1 must be very low in this cell line. These results, along with the cAMP-induced enhancement of IGF-I-dependent MAP kinase activation (Figure 5), are mainly the predicted consequence of the cAMP enhancement of IGF-I-dependent tyrosine phosphorylation of 66 kDa Shc.

Tyrosine-phosphorylated IRS-1 and IRS-2 associate with the p85 regulatory subunit of PI 3-kinase, resulting in activation of the PI 3-kinase cascade, which also controls various cell functions [31]. In the present study, pretreatment with cAMP decreased the amount of p85 subunit bound to phosphorylated IRS-1, but increased its binding to IRS-2 (Figures 6A and 6B). We could detect large amounts of PI 3-kinase p85 subunit in the supernatant after immunodepletion of IRS-1 and IRS-2 from the lysates of cells pretreated with TSH or dibutyl cAMP (results not shown). These results indicate that the decrease in p85 subunit association with IRS-1 was not due to an increased interaction between p85 subunit and IRS-2. These changes in the amounts of the p85 subunit associated with IRS-1 or IRS-2 reflected well the PI 3-kinase activity that was bound to IRS-1 or IRS-2 (Figures 6 and 7). Furthermore, an IGF-I-dependent increase in PI 3-kinase activity in the immunoprecipitates, as detected with an anti-phosphotyrosine antibody, was amplified by cAMP pretreatment (Figure 7C), suggesting that total PI 3-kinase activity was increased through changes in IRS-2 tyrosine phosphorylation. Although the mechanisms leading to these changes remain to be elucidated, our new observations also support our hypothesis that IRS-1 and IRS-2 possess different roles in the mediation of IGF-I bioactivities in FRTL-5 cells.

Finally, we tested the effects of the presence of an inhibitor of each signalling pathway during IGF-I treatment on DNA synthesis in FRTL-5 cells pretreated with cAMP and then treated with IGF-I. cAMP-dependent potentiation of DNA synthesis induced by IGF-I was partially, but significantly, depressed by the MEK inhibitor PD98059 (Figure 8A). Thus potentiation by cAMP of the MAP kinase cascade in response to IGF-I could mediate, in part, the potentiation of cell growth

stimulated by cAMP and IGF-I. In addition, LY294002, a PI 3-kinase inhibitor, completely abolished IGF-I-dependent DNA synthesis, whether or not there was cAMP pretreatment (Figure 8B). From these results, we conclude that PI 3-kinase activation is essential to mediate IGF-I-dependent cell growth in this cell line.

In the present study we have focused on the cAMP-dependent enhancement of IGF-I-dependent signals through changes in tyrosine phosphorylation, and shown this to be an important mechanism of the cross-talk between cAMP and growth factors. Other groups have shown that a cAMP stimulus changes the amount or activity of cyclin/Cdk (cyclin-dependent kinase)/Cdk inhibitor systems in FRTL-5 cells [46]. These mechanisms may be complementary in amplifying the traverse of cells through the cell cycle in response to IGF-I.

In summary, we have demonstrated that, in FRTL-5 cells, pretreatment with cAMP enhances the IGF-I-dependent tyrosine phosphorylation of IRS-2 and 66 kDa Shc. Our results suggest that the interaction between cAMP-dependent and IGF-I-dependent pathways leads to an augmentation of cell proliferation, which is mediated, at least in part, through the MAP kinase and PI 3-kinase signalling pathways. These effects are mediated by changes in the tyrosine phosphorylation of IGF-I receptor substrates, including IRS-2 and Shc.

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