LRP-1/TβR-V mediates TGF-β1-induced growth inhibition in CHO cells

Wen-Fang Tseng[a,b], Shuan Shian Huang[b], Jung San Huang[b,*]

[a] *Institute of Life Science, National Defense Medical Center, Taipei, Taiwan*
[b] *Department of Biochemistry and Molecular Biology, Saint Louis University School of Medicine, 1402 South Grand Boulevard, St. Louis, MO 63104, USA*

Received 8 January 2004; accepted 6 February 2004

First published online 27 February 2004

Edited by Veli-Pekka Lehto

Abstract The type V transforming growth factor-β (TGF-β) receptor (TβR-V) is hypothesized to be involved in cellular growth inhibition by TGF-β_1. Recently, TβR-V was found to be identical to low density lipoprotein receptor-related protein-1 (LRP-1). Here we demonstrate that TGF-β_1 inhibits growth of wild-type CHO cells but not LRP-1-deficient mutant cells (CHO-LRP-1$^-$ cells). Stable transfection of CHO-LRP-1$^-$ cells with LRP-1 cDNA restores the wild-type morphology and the sensitivity to growth inhibition by TGF-β_1. In addition, overexpression of LRP-1 minireceptors exerts a dominant negative effect and attenuates the growth inhibitory response to TGF-β_1 in wild-type CHO cells. These results suggest that LRP-1/TβR-V is critical for TGF-β_1-mediated growth inhibition in CHO cells.

Key words: Transforming growth factor-β_1; Growth inhibition; Type V transforming growth factor-β receptor; Lipoprotein receptor-related protein-1; CHO cell; Lipoprotein receptor-related protein-1 minireceptor

1. Introduction

The type V transforming growth factor-β (TGF-β) receptor (TβR-V) was identified as a high-molecular-weight receptor which co-expresses with other TGF-β receptor types in most cell types [1–3]. It was subsequently found to be identical to the insulin-like growth factor-binding protein-3 (IGFBP-3) receptor which mediates the IGF-independent growth inhibitory response upon stimulation of IGFBP-3 in responsive cells [4,5]. Many carcinoma cells produce little or no TβR-V and do not respond to growth inhibition induced by IGFBP-3 or TGF-β. Loss of TβR-V is hypothesized to contribute to the malignant phenotype. Surprisingly, TβR-V was recently also found to be identical to low density lipoprotein receptor-related protein-1 (LRP-1) [6]. This finding was unexpected because LRP-1 has generally been recognized as an endocytic receptor involved in plasma clearance and cellular degradation of ligands [7–10].

LRP-1 is synthesized as a 600-kDa type I membrane glycoprotein. It can be cleaved into a 515-kDa heavy chain and an 85-kDa light chain upon proteolytic processing after biosynthesis [7–11]. These two chains are held together by non-covalent forces. Both the two-chain and intact molecules of LRP-1 are present on the cell surface [6]. The light chain of LRP-1 contains a transmembrane domain and a cytoplasmic domain which includes endocytic motifs and the phosphorylation sites for cAMP-dependent protein kinase and the platelet-derived growth factor-β-type receptor [12–15]. The heavy chain of LRP-1 consists of several structural units: ligand-binding complement-type cysteine-rich repeats, EGF-like cysteine-rich repeats and YWTD motifs. These repeats are arranged in four clusters containing two, eight, 10 and 11 repeats which are referred to as domains I, II, III and IV, respectively. Domains II and IV are responsible for binding most known LRP-1 ligands [7–11].

LRP-1 is identical to the activated α_2-microglobulin (α_2M*) receptor [16]. α_2M* has been shown to reduce *N*-methyl-D-aspartic acid receptor-mediated Ca^{2+} influx in neurons and stimulate a rise in cytoplasmic Ca^{2+} in 1-LN human prostate cancer cells and murine macrophages [17–20]. Increasing evidence indicates that LRP-1 is capable of mediating signals other than Ca^{2+} influx [21]. The identification of TβR-V as LRP-1 means that LRP-1 has a previously unreported growth regulatory function [6]. This newly identified function of LRP-1 may explain the embryonic lethal phenotype of the LRP-1 gene null mutation in animals [22]. Since LRP-1/TβR-V seems to be quite significant in animal biology, we decided to investigate its role in the growth inhibitory response to TGF-β_1 in Chinese hamster ovary (CHO) cells. Wild-type CHO cells (CHO-K1 cells) and mutant cells which are deficient in LRP-1 (CHO-LRP-1$^-$ cells) [23] provide a model cell system to test the role of LRP-1 in the growth inhibitory response to TGF-β_1. In this communication, we demonstrate that LRP-1/TβR-V is required for growth inhibition induced by TGF-β_1 in the CHO cell system.

2. Materials and methods

2.1. Materials

$Na^{125}I$ (17 Ci/mg) and [methyl-^{3}H]thymidine (67 Ci/mmol) were purchased from ICN Biochemical (Irvine, CA, USA). Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium, hygromycin, G418, murine monoclonal antibody to HA peptide (YPYDVPDYA) and chloramine T were obtained from Sigma (St. Louis, MO, USA). Precision protein standards and protein molecular weight markers were obtained from Bio-Rad (Hercules, CA, USA) and Promega (Madison, WI, USA). Anti-human LRP light chain serum (carboxy-terminal 15-residue peptide) was prepared as described previously [6]. Rabbit anti-LRP-1 (whole molecule) serum was provided by Dr. Dudley K. Strickland, Department of Vascular Biology, American Red Cross, Rockville, MD, USA. GST-RAP (a fusion protein of glutathione *S*-transferase and receptor-associated protein) was expressed in *Escherichia coli* using pGEX-KG-RAP (6.4 kb) plasmid and purified according to the procedure of Herz et al. [24]. pGEX-KG-RAP, pcDNA3.1(-)neo and pcDNA3.1(-)neo-LRP-1 plasmid

*Corresponding author. Fax: (1)-314-977 9205.
E-mail address: huangjs@slu.edu (J.S. Huang).

0014-5793/04/$30.00
doi:10.1016/S0014-5793(04)00185-1

were provided by Dr. Joachim Herz, Department of Molecular Genetics, Texas Southwestern Medical Center, Dallas, TX, USA. pcDNA3.0 and lipofectamine 2000 were obtained from Invitrogen (Carlsbad, CA, USA). Disuccinimidyl suberate was obtained from Pierce (Rockford, IL, USA). Human TGF-β_1 was obtained from Austral Biologicals (Santa Clara, CA, USA). Human IGFBP-3 (expressed in *E. coli*; MW ~35 000) was obtained from Upstate (Charlottesville, VA, USA). CHO-K1 cells were obtained from American Type Culture Collection (Rockville, MD, USA). CHO-LRP-1$^-$ cells [23] were provided by Dr. Guejun Bu, Department of Pediatrics and Cell Biology and Physiology, Washington University, St. Louis, MO, USA. α_2M* was prepared as described previously [25,26].

2.2. Preparation of cDNA constructs

The cDNA of LRP-1 was obtained by digestion of pcDNA3.1-LRP-1 [6] with *Eco*RV. The LRP-1 cDNA fragment was then ligated into the *Xho*I site of the pCXN vector after addition of phosphorylated *Xho*I linkers and *Xho*I digestion. LRP-1 minireceptor (mLRP) cDNAs contained three parts: encoding signal peptide with an attached HA tag, the ligand-binding minidomain (domain I, II, II or IV) and the LRP-1 light chain. These mLRP cDNAs were prepared according to the procedures published by Obermoeller-McCormick et al. [27]. Briefly, the relevant DNA sequences were synthesized by polymerase chain reaction (PCR) using primers as described previously [27]. The PCR products were purified and ligated into the vector pcDNA 3.0. These constructs were named pcDNA 3.0-mLRPI, pcDNA 3.0-mLRPII, pcDNA 3.0-mLRPIII and pcDNA 3.0-mLRPIV for constructs containing cDNAs of mLRPI, mLRPII, mLRPIII, and mLRPIV, respectively.

2.3. Transfection

Cells were grown on 24-well dishes (2×10^5/well) in DMEM/Ham's F-12 medium containing 10% fetal calf serum. For LRP-1 cDNA transfection, CHO-LRP-1$^-$ cells [23] were transfected with 0.8 μg pCXN-LRP-1+0.04 μg pcDNA 3.1-Hygro or 0.8 μg pCXN+0.04 μg pcDNA 3.1-Hygro (vectors) using lipofectamine 2000 [28]. Hygromycin-resistant cells were selected with 600 μg/ml of hygromycin and 1000 μg/ml of G418 in DMEM/Ham's F-12 medium containing 10% fetal calf serum. CHO-LRP-1$^-$ cells already expressed the *neo* gene [27]. G418 included in the selection medium was used to amplify the LRP-1 cDNA insert in the pCXN vector in transfected cells [29]. The selected clones were named CHO-LRP-1$^-$/LRP-1 and CHO-LRP-1$^-$/vector cells, respectively. Four to six clones were selected for each cell type. For mLRP cDNA transfection, CHO-K1 and CHO-LRP-1$^-$ cells were transfected with 0.8 μg of pcDNA 3.1-mLRPs or vector only using the same lipofectamine 2000 method. The transfected cells were selected with 1000 μg/ml of G418. The selected clones were named CHO-K1/mLRPI, CHO-LRP-1$^-$/mLRPI, CHO-K1/mLRPII, CHO-LRP-1$^-$/mLRPII, CHO-K1/mLRPIII, CHO-LRP-1$^-$/mLRPIII, CHO-K1/mLRPIV and CHO-LRP-1$^-$/mLRPIV cells, which expressed mLRPI, mLRPII, mLRPIII and mLRPIV, respectively. CHO-K1/vector and CHO-LRP-1$^-$/vector cells were CHO-K1 and CHO-LRP-1$^-$ cells stably transfected with vector only, respectively. Four to six clones were selected for each cell type. These cells were maintained in the same medium containing 400 μg/ml of G418.

2.4. [^{125}I]IGFBP-3 affinity labeling

Cells were affinity-labeled with [^{125}I]IGFBP-3 according to published procedures [4,5]. The [^{125}I]IGFBP-3 affinity-labeled TβR-V was analyzed by 5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and autoradiography.

2.5. Western blot analysis

Cells were lysed with 1% Triton X-100. The cell lysates were subjected to Western blot analysis using murine monoclonal antibody to HA, and rabbit antiserum to the light chain of LRP-1 or to the whole molecule of LRP-1 followed by 5 or 10% SDS–PAGE. The antigen band on the blot was visualized using the ECL system and quantitated using Image Quant.

2.6. [^{125}I]α_2M* binding analysis

[^{125}I]α_2M* was prepared using methylamine-activated human α_2M* and the chloramine T method as described previously [25,26]. Cells were incubated with 10 nM of [^{125}I]α_2M* in the presence or absence of 15 μg/ml of GST-RAP. The presence of GST-RAP was used to estimate non-specific binding of [^{125}I]α_2M*. After 2.5 h at 0°C, the binding was determined. Specific binding was determined by subtracting non-specific binding from total binding.

2.7. [Methyl-^{3}H]thymidine incorporation assay

Cells grown on 48-well clustered dishes (0.5×10^5/well) in DMEM/Ham's F-12 medium containing 0.1% fetal calf serum were treated with increasing concentrations of TGF-β_1 [6]. After incubation at 37°C for 18 h, the cells were washed twice with 10% trichloroacetic acid and once with 0.5 ml of ethanol:ether (2:1, v/v) and dissolved in 0.2 N NaOH for scintillation counting.

2.8. Cell growth assay

The cell growth assay was carried out as previously described [6]. Cells were treated with or without 100 pM of TGF-β_1 in DMEM/Ham's F12 medium containing 1% fetal calf serum. Additional TGF-β_1 (100 pM) or solvent vehicle was added to the medium in the second day of incubation. The cell number was counted 1 day later using a hematocytometer.

2.9. Endocytosis

Endocytosis of cell surface-bound [^{125}I]α_2M* in CHO-K1/vector and CHO-K1 cells stably expressing mLRPs was carried out as described previously [27,30]. Cells grown on 35-mm Petri dishes were incubated with 10 nM [^{125}I]α_2M* in the presence or absence of 15 μg/ml GST-RAP in DMEM/Ham's F-12 medium (bicarbonate-free) containing 0.1% bovine serum albumin. After 2 h at 0°C, cells were washed with cold buffer (50 mM HEPES, pH 7.4, 128 mM NaCl, 5 mM $MgSO_4$ and 1.2 mM $CaCl_2$). Endocytosis of cell surface-bound [^{125}I]α_2M* was started by addition of 37°C-prewarmed DMEM/Ham's F-12 medium. After various time periods at 37°C, cells were treated with 0.5 mg/ml of trypsin in phosphate-buffered saline containing 0.5 mM EDTA at 0°C for 20 min. The trypsin-released and cell-associated radioactivity were determined and represented cell surface-bound and internalized [^{125}I]α_2M*, respectively.

3. Results

3.1. TGF-β_1 inhibits growth of CHO-K1 cells but not CHO-LRP-1$^-$ cells

CHO-LRP-1$^-$ cells were derived from CHO-K1 cells by mutagenesis of the cells followed by selection with *Pseudomonas* exotoxin A [23]. These cells did not express detectable endogenous LRP-1. The absence of LRP-1/TβR-V in CHO-LRP-1$^-$ cells was demonstrated by [^{125}I]IGFBP-3 affinity labeling (Fig. 1A), Western blot analysis using antiserum to the light chain of LRP-1 (Fig. 1B) and [^{125}I]α_2M* binding analysis (Fig. 1C). CHO-LRP-1$^-$ cells express as many TGF-β type I, type II and type III receptors (TβR-I, TβR-II and TβR-III) as the wild-type cells (CHO-K1 cells) as determined by [^{125}I]TGF-β_1 affinity labeling (data not shown). In CHO-K1 cells, TβR-V cannot be detected by [^{125}I]TGF-β_1 affinity labeling because [^{125}I]TGF-β_1 affinity-labeled TβR-III (which migrates as a broad high-molecular-weight band on SDS–PAGE) hinders the detection of [^{125}I]TGF-β_1 affinity-labeled TβR-V on SDS–PAGE as observed in several cell types [2]. [^{125}I]IGFBP-3 affinity labeling was, therefore, used to detect cell surface TβR-V [3,4]. The light chain of LRP-1, which contains the transmembrane and cytoplasmic domains, is stable and appropriate to use as an indicator for the measurement of LRP-1 expression. The recovery of the LRP-1 heavy chain varies with experimental conditions because it associates non-covalently with the light chain [6]. For this reason, antiserum to the light chain of LRP-1 was used to identify LRP-1 [6]. α_2M* is a well-characterized ligand for LRP-1 which is ideal for measurement of cell surface expression of LRP-1 [16]. At the steady state, >95% of LRP-1 is present in the

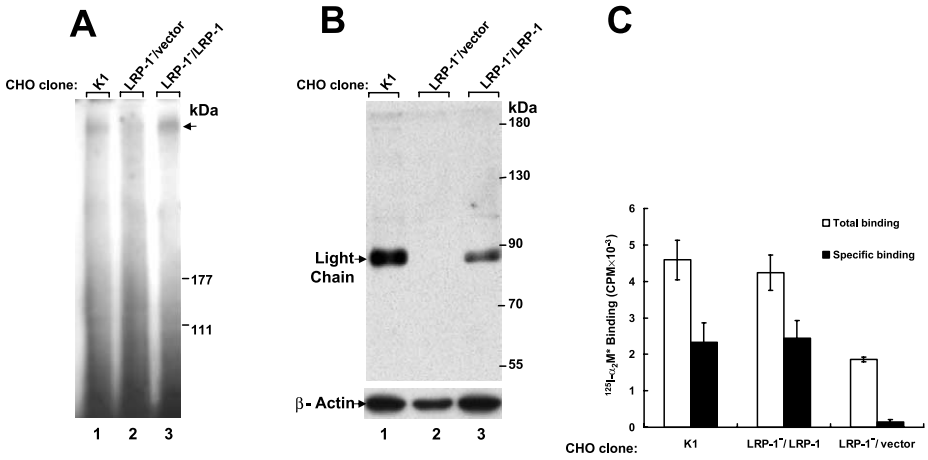

Fig. 1. [^{125}I]IGFBP-3 affinity labeling (A), Western blot analysis (B) and [^{125}I]α_2M* binding analysis (C) in CHO-K1, CHO-LRP-1$^-$/vector and CHO-LRP-1$^-$/LRP-1 cells. A: CHO-K1 (lane 1), CHO-LRP-1$^-$/vector (lane 2) and CHO-LRP-1$^-$/LRP-1 (lane 3) cells were affinity-labeled with [^{125}I]IGFBP-3 according to published procedures [4,5]. The [^{125}I]IGFBP-3 affinity-labeled TβR-V was analyzed by 5% SDS–PAGE and autoradiography. The arrow indicates the location of the [^{125}I]IGFBP-3–TβR-V complex. B: Cell lysates of CHO-K1 (lane 1), CHO-LRP-1$^-$/vector (lane 2) and CHO-LRP-1$^-$/LRP-1 (lane 3) cells were subjected to Western blot analysis using antisera to the light chain of LRP-1 and β-actin. The arrow indicates the locations of the light chain of LRP-1 and β-actin. The ratio of the LRP-1 light chain and β-actin levels in CHO-K1 cells was taken as 1. This ratio in CHO-LRP-1$^-$/LRP-1 cells was estimated to be 0.6 ± 0.2 ($n = 3$). C: CHO-K1, CHO-LRP-1$^-$/LRP-1 and CHO-LRP-1$^-$/vector cells were incubated with 10 nM [^{125}I]α_2M* in the presence and absence of 15 μg/ml RAP (for estimating non-specific binding) at 0°C for 2.5 h. The specific binding of [^{125}I]α_2M* was estimated by subtracting non-specific binding from total binding.

intracellular compartments [7–11]. As shown in Fig. 1A, the [^{125}I]IGFBP-3 affinity-labeled TβR-V complex was detected in CHO-K1 cells (lane 1) but not in CHO-LRP-1$^-$ cells (lane 2). CHO-K1 cells expressed LRP-1 whereas CHO-LRP-1$^-$ cells did not (Fig. 1B, lane 1 versus lane 2). CHO-K1 cells also showed specific binding of [^{125}I]α_2M* to LRP-1, whereas CHO-LRP-1$^-$/vector cells did not (Fig. 1C).

In preliminary studies, we found that unlike epithelial cells, CHO-K1 cells exhibited growth inhibition by TGF-β_1 but not by IGFBP-3. To compare the effect of TGF-β_1 on both DNA synthesis and cell growth between CHO-K1 and CHO-LRP-1$^-$ cells, cells were incubated with increasing concentrations of TGF-β_1 for DNA synthesis determination or with 100 pM TGF-β_1 for cell growth assay. After 18 h at 37°C, DNA synthesis was determined by measuring [methyl-^{3}H]thymidine incorporation into cellular DNA. For the cell growth assay, cell number was counted after a 3-day incubation with or without TGF-β_1. As shown in Fig. 2A,B, TGF-β_1 at 20 pM and 100 pM inhibited DNA synthesis and cell growth of CHO-K1 cells by ~30% and ~60%, respectively. By contrast, it stimulated DNA synthesis of CHO-LRP-1$^-$ cells though it did not significantly affect growth of these cells in the cell growth assay. These results suggest that although TGF-β is a growth inhibitor for CHO-K1 cells, it fails to inhibit growth in CHO-LRP-1$^-$/vector cells.

3.2. *Stable transfection of CHO-LRP-1$^-$ cells with LRP-1 cDNA restores the wild-type morphology and the growth inhibitory response to TGF-β_1*

TβR-V has recently been shown to mediate the growth inhibitory response to TGF-β_1 in concert with other TGF-β receptors [3,6]. It is likely that the lack of the growth inhibitory response to TGF-β_1 in CHO-LRP-1$^-$ cells is due to the absence of LRP-1 in these cells. To test this, CHO-LRP-1$^-$ cells were stably transfected with LRP cDNA in pCXN vector containing the *neo* gene or vector only and selected with G418. The presence of G418 in the selection medium selected for expression and amplification of the *neo*-containing vector,

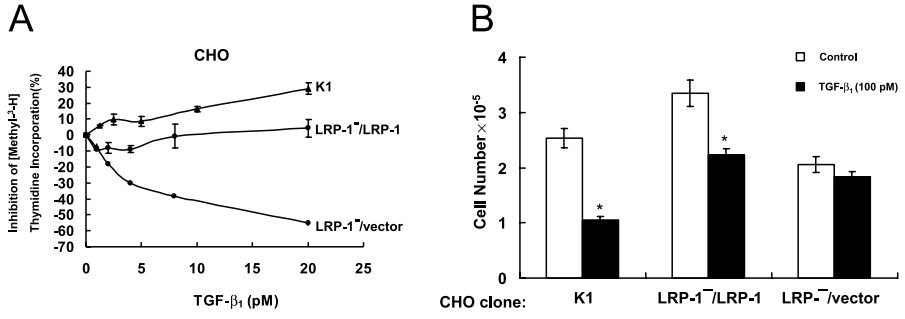

Fig. 2. Effect of TGF-β_1 on DNA synthesis (A) and cell growth (B) in CHO-K1, CHO-LRP-1/vector and CHO-LRP-1$^-$/LRP-1 cells. CHO-K1, CHO-LRP-1$^-$/LRP-1 and CHO-LRP-1$^-$/vector cells were incubated with increasing concentrations of TGF-β_1 as indicated for DNA synthesis assays (A) or with TGF-β_1 (0 and 100 pM) for cell growth assays (B). After 18 h at 37°C, DNA synthesis was determined by measurement of [methyl-^{3}H]thymidine incorporation into cellular DNA. The [methyl-^{3}H]thymidine incorporation in cells treated without TGF-β_1 was taken as 100%. For cell growth assays, cells were counted after 3 days in culture. Data from CHO-K1 and CHO-LRP-1$^-$/LRP-1 cells treated with TGF-β_1 were compared with data from these cells treated without TGF-β_1 by Student's *t*-test ($^*P < 0.001$). Data are representative of four similar experiments.

pCXN and simultaneous amplification of the LRP-1 cDNA insert in the pCXN vector which was integrated into the targeted cell genome [29]. The selected representative clones were named CHO-LRP-1$^-$/LRP-1 and CHO-LRP-1$^-$/vector, respectively. As shown in Fig. 1, CHO-LRP-1$^-$/LRP-1 expressed LRP-1 at a level comparable to that in wild-type cells (CHO-K1 cells) as determined by [^{125}I]IGFBP-3 affinity labeling (Fig. 1A, lane 3 versus lane 1), Western blot analysis (Fig. 1B, lane 3 versus lane 1) and [^{125}I]α_2M* binding analysis (Fig. 1C). CHO-LRP-1$^-$/vector cells did not produce measurable LRP-1 in these three assays. CHO-LRP-1$^-$/LRP-1 cells exhibited a round shape, similar to that seen in wild-type cells (CHO-K1 cells) (Fig. 3). By contrast, like untransfected CHO-LRP-1$^-$ cells, CHO-LRP-1$^-$/vector cells had spindle shape morphology (Fig. 3).

We then determined the effect of TGF-β_1 on DNA synthesis in CHO-LRP-1$^-$/LRP-1, CHO-LRP-1$^-$/vector and CHO-K1 cells. As shown in Fig. 2A, TGF-β_1 inhibited DNA synthesis in CHO-LRP-1$^-$/LRP-1 and CHO-K1 cells. This is in contrast to the TGF-β_1-stimulated DNA synthesis seen in CHO-LRP-1$^-$/vector cells. At 20 pM, TGF-β_1 inhibited DNA synthesis by ~5% and ~30% in CHO-LRP-1$^-$/LRP-1 and CHO-K1 cells, respectively, whereas it stimulated DNA synthesis by ~50% in CHO-LRP-1$^-$/vector cells. The growth inhibitory response to TGF-β_1 in these CHO cells was also determined by directly counting cell number after the cells had been treated with TGF-β_1 for 3 days. As shown in Fig. 2B, TGF-β_1 (100 pM) inhibited growth of CHO-K1 and CHO-LRP-1$^-$/LRP-1 cells by ~60 and ~30%, respectively but did not significantly affect growth of CHO-LRP-1$^-$/vector cells. The magnitude of the growth inhibitory response to TGF-β_1 as determined by measurement of DNA synthesis and cell number in CHO-LRP-1$^-$/LRP-1 cells appeared to be less than that in CHO-K1 cells. This partial rescue of TGF-β_1-mediated growth inhibition may be due to unidentified alterations in the growth inhibitory response-related cellular events in CHO-LRP-1$^-$ cells. CHO-LRP-1$^-$ cells were originally produced by mutagenesis and selection with *Pseudomonas* exotoxin A. These results suggest that stable transfection of CHO-LRP-1$^-$ cells with LRP-1 cDNA restores the wild-type phenotype and the sensitivity to growth inhibition by TGF-β_1.

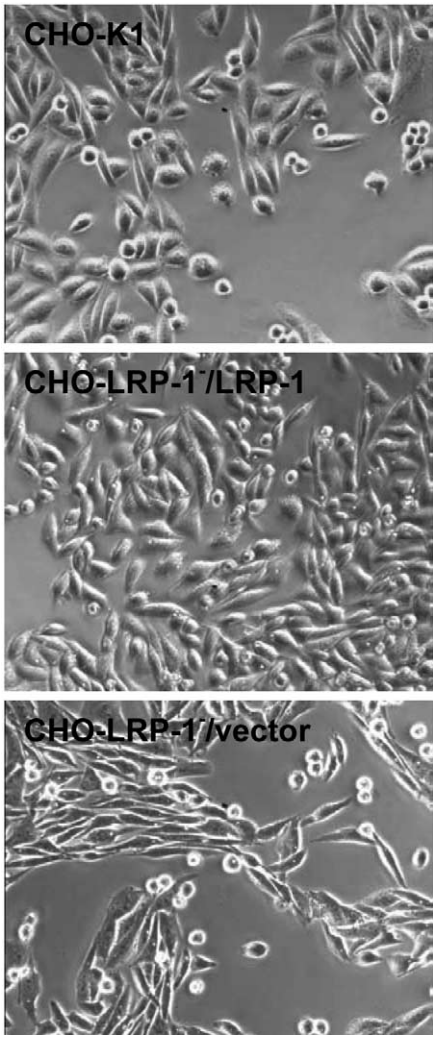

Fig. 3. Microscopic morphology of CHO-K1, CHO-LRP-1$^-$/vector and CHO-LRP-1$^-$/LRP-1 cells.

3.3. Overexpression of mLRPs attenuates the growth inhibitory response to TGF-β_1 in CHO-K1 cells

We hypothesized that LRP-1 not only binds TGF-β_1 but also is involved in mediating signaling which leads to growth inhibition induced by TGF-β_1 (in concert with other TGF-β receptor types). If this hypothesis is correct, mLRPs containing individual cell surface ligand-binding domains [27] might have the potential to function as dominant negative mutants when they are overexpressed in cells expressing endogenous LRP-1. To test this hypothesis, CHO-K1 cells were stably transfected with HA-tagged mLRPI, II, III and IV cDNAs and vector only. The clones selected were named CHO-K1/mLRPI, CHO-K1/mLRPII, CHO-K1/mLRPIII and CHO-K1/mLRPIV, respectively. These cells expressed the products of these cDNA constructs of mLRPs with molecular masses of intact mLRPs (~120 kDa for mLRPI, ~200 kDa for mLRPII, 230 kDa for mLRPIII and ~200 kDa for mLRPIV) and of the ligand binding domain or the heavy chain of mLRPs (~40 kDa for mLRPI, ~120 kDa for mLRPII, ~150 kDa for mLRPIII and ~120 kDa for mLRPIV) (Fig. 4A, lanes 2 and 5–7) as described previously [27]. These mLRPs all had the same light chain (85 kDa) as endogenous LRP-1 did. The expression levels for all mLRPs were estimated to be ~2.5–3.5-fold higher than that of endogenous LRP-1 as determined by Western blot analysis using antiserum to the light chain of LRP-1 (Fig. 4B, lanes 2–5 versus lane 1). The expression levels of the heavy chain of endogenous LRP-1 in these CHO-K1 cells stably expressing mLRPs were comparable to that in CHO-K1/vector cells (Fig. 4B, lanes 2–5 versus lane 1). This suggests that overexpression of mLRPs does not greatly affect the expression of endogenous LRP-1. Among these mLRPs, only mLRPII and mLRPIV exhibited [^{125}I]IGFBP-3 binding as determined by [^{125}I]IGFBP-3 affinity labeling (Fig. 4C, lanes 2 and 3). mLRPI and mLRPIII did not have detectable [^{125}I]IGFBP-3 binding (data not shown). Overexpression of these mLRPs all attenuated the growth inhibitory response (as determined by measurement of DNA synthesis and cell number) to TGF-β_1 in CHO-K1 cells (Fig. 5A,B). By contrast, CHO-K1 cells stably transfected with vector only responded as untransfected CHO-K1 cells did (Fig. 5A, versus Fig. 2A). These mLRPs

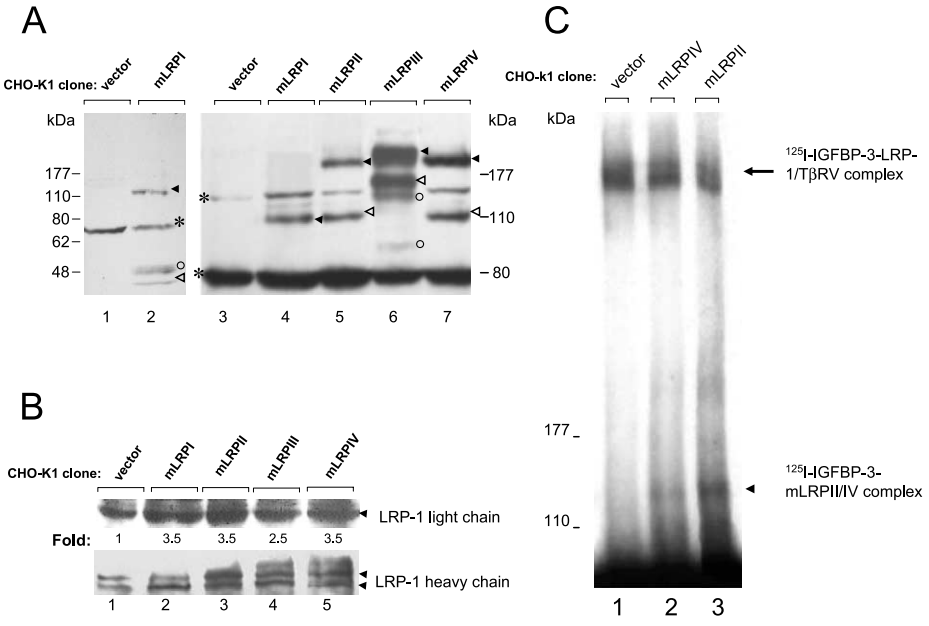

Fig. 4. Western blot analyses of mLRPs (A) and of the light chain and the heavy chain of LRP-1 (B) and [^{125}I]IGFBP-3 affinity labeling (C) in CHO-K-1 cells stably expressing mLRPs. A: Cell lysates from CHO-K1/vector cells and CHO-K1 cells stably expressing mLRPs (mLRPI, mLRPII, mLRPIII and mLRPIV) were analyzed by Western blot analysis using murine monoclonal antibody to HA following 10% (lanes 1 and 2) or 5% (lanes 3–7) SDS–PAGE. The closed arrowhead indicates the locations of intact mLRPs (~120 kDa for mLRPI, ~200 kDa for mLRPII, ~230 kDa for mLRPIII and ~200 kDa for mLRPIV) (lanes 4–7). The open arrowhead indicates the locations of the mLRP light chains (~40 kDa for mLRPI, ~120 kDa for mLRPII, ~150 kDa for mLRPIII and ~120 kDa for mLRPIV) (lanes 2, 5, 6 and 7). The asterisk indicates the locations of non-specific bands. The open circle indicates the locations of the degradation products of mLRPI and mLRPIII, which were also reported previously [27]. B: The cell lysates from CHO-K1/vector and CHO-K1 cells stably expressing mLRPs were subjected to Western blot analysis using antiserum to LRP-1. The arrowhead indicates the locations of the light chain and the heavy chain of LRP-1. The relative amounts of the LRP-1 heavy chain (from endogenous LRP-1) and of the LRP-1 light chain (from endogenous LRP-1+mLRPs) in these CHO-K1 cells stably expressing mLRPI were determined using Image Quant. The relative amounts of the light chain of LRP-1 were estimated to be 1, ~3.5, ~3.5, ~2.5 and ~3.0 in CHO-K1/vector, CHO-K1/mLRPI, CHO-K1/mLRPII, CHO-K1/mLRPIII and CHO-K1/mLRPIV cells, respectively. C: CHO-K1 /vector (lane 1), CHO-K1/mLRPII (lane 3) and CHO-K1/mLRPIV (lane 2) cells were incubated with 6 nM [^{125}I]IGFBP-3 for 25 h at 0°C. [^{125}I]IGFBP-3 affinity labeling was then performed. The arrow indicates the location of the [^{125}I]IGFBP-3–LRP-1/TβR-V complex. The arrowhead indicates the location of either the [^{125}I]IGFBP-3–mLRPII or the [^{125}I]IGFBP-3–mLRPIV complex (MW ~150 000).

did not have detectable ability to mediate growth inhibition by TGF-β_1. As shown in Fig. 5B, CHO-LRP-1$^-$ cells stably transfected with mLRPIV cDNA (CHO-LRP-1$^-$/mLRPIV cells) did not respond to TGF-β_1-induced growth inhibition. Similar results were also obtained in CHO-LRP-1$^-$ cells stably expressing mLRPI, II and III (data not shown). These results suggest these mLRPs are capable of functioning as dominant negative mutants in CHO-K1 cells.

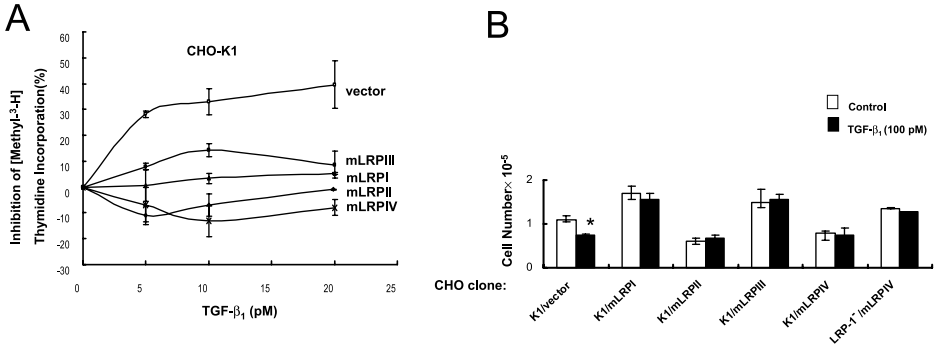

Fig. 5. Effect of TGF-β_1 on DNA synthesis (A) and cell growth (B) in CHO-K1/vector and CHO-LRP-1$^-$ cells stably expressing mLRPs. CHO-K1 cells stably expressing mLRPs and vector only (K1/mLRPI, K1/mLRPII, K1/mLRPIII, K1/mLRPIV and K1/vector) and CHO-LRP-1$^-$ cells stably expressing mLRPIV (LRP-1$^-$/mLRPIV cells) were treated with increasing concentrations of TGF-β_1 as indicated for DNA synthesis assays (A) or with 100 pM of TGF-β_1 for cell growth assay (B). After 18 h at 37°C, [methyl-^{3}H]thymidine incorporation into cellular DNA was determined. The [methyl-^{3}H]thymidine incorporation in cells treated without TGF-β_1 was taken as 100%. In the cell growth assay, the cell number was counted after a 3-day incubation. All data points from CHO-K1/mLRPI, CHO-K1/mLRPII, CHO-K1/mLRPIII and CHO-K1/mLRPIV cells (A) were statistically significant when compared with those in CHO-K1/vector cells (Student's t-test, $P < 0.05$–0.001). Data of the cell growth assay (B) from CHO-K1/vector cells treated with TGF-β_1 were compared with data from these cells treated without TGF-β_1 by Student's t-test (*$P < 0.05$). Data are representative of three similar experiments.

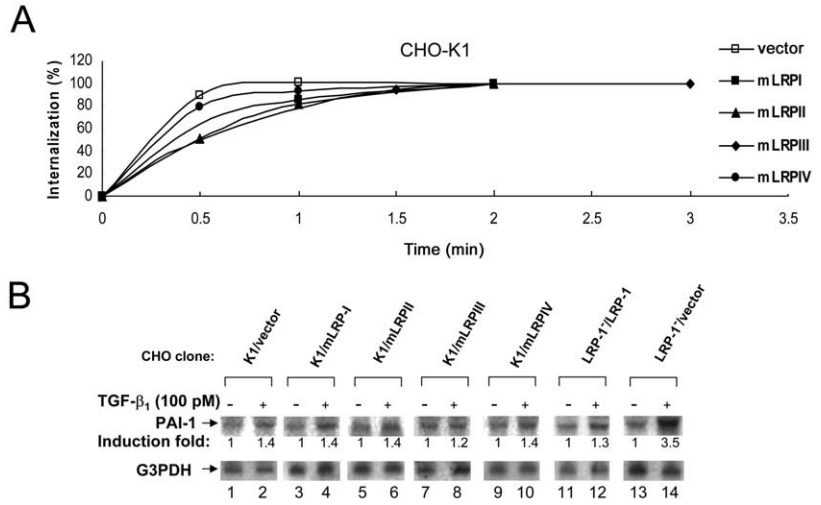

Fig. 6. Cell surface [^{125}I]α_2M* endocytosis (A) and TGF-β_1-stimulated PAI-1 expression (B) in CHO-K1 cells stably expressing mLRPs. A: CHO-K1 cells stably expressing vector, mLRPI, mLRPII, mLRPIII and mLRPIV were incubated with 10 nM [^{125}I]α_2M* in the presence or absence of GST-RAP (15 μg/ml). After 2.5 h at 0°C, the endocytosis of cell surface [^{125}I]α_2M* was carried out at 37°C for different time periods as indicated. Cell surface-associated and internalized [^{125}I]α_2M* were determined after trypsin digestion of cells at 0°C. The trypsin-released and cell-associated radioactivity represented cell surface-bound and internalized [^{125}I]α_2M*, respectively. The presence of GST-RAP was used to determine non-specific binding. Internalization of cell surface [^{125}I]α_2M* reached the maximum after a 2–3-min incubation at 37°C, which represented ~80–90% of the specific binding prior to incubation at 37°C. The maximum was taken as 100% of internalization. B: CHO-K1 cells stably expressing vector only and mLRPs (K1/vector, K1/mLRPI, K1/mLRPII, and K1/mLRPIII, and K1/mLRPIV cells) and CHO-LRP-1$^-$ cells stably expressing LRP-1 and vector only (LRP-1$^-$/LRP-1 and LRP-1$^-$/vector cells) were treated with or without TGF-β_1 (100 pM). After 2 h at 37°C, Northern blot analysis of PAI-1 and G3PDH were performed. The intensities of the transcripts were determined by a PhosphoImager. The ratio of PAI-1/G3PDH was taken as 1-fold of PAI-1 expression in cells treated without TGF-β_1. The TGF-β_1-induced PAI-1 expression was estimated to be 1.4-, 1.4-, 1.4-, 1.2-, 1.4-, 1.3- and 3.5-fold in CHO-K1/vector, CHO-K1/mLRPI, CHO-K1/mLRPII, CHO-K1/mLRPIII, CHO-K1/mLRPIV cells, CHO-LRP-1$^-$/LRP-1 and CHO-LRP-1$^-$/vector cells (lanes 1/2–13/14, respectively).

3.4. *Overexpression of mLRPs does not significantly affect the endocytosis rate of endogenous LRP-1 and TGF-β_1-induced PAI-1 expression in CHO-K1 cells*

The finding that various mLRPs containing individual domains all affect the growth inhibitory response to TGF-β_1 was unexpected. Although the binding site(s) for TGF-β_1 in the LRP-1 molecule has not been identified, it is possible but unlikely that all four mLRPs are capable of binding TGF-β_1 and competing with endogenous LRP-1 for binding TGF-β_1. Since LRP-1 is a known endocytic receptor, it would also be important to see whether overexpression of mLRPs alters the endocytic rate of cell surface endogenous LRP-1. The endocytosis rate of cell surface endogenous LRP-1 in these CHO-K1 cells stably expressing mLRPs was measured using [^{125}I]α_2M* as ligand as described previously [27,30]. As shown in Fig. 6A, CHO-K1/mLRPI, CHO-K1/mLRPII, CHO-K1/mLRPIII and CHO-K1/mLRPIV cells exhibited similar endocytosis rates of cell surface endogenous LRP-1 ($t_{1/2}$ ~ 20–30 s) when compared with that of cell surface LRP-1 in CHO-K1/vector cells ($t_{1/2}$ ~ 20–30 s) [27,30].

To determine the effect of mLRP overexpression on TβR-I/TβR-II/Smad2/3/4 signaling, we examined TGF-β_1-induced PAI-1 expression in CHO-K1 cells stably expressing mLRPs. TGF-β-induced PAI-1 expression is known to be mainly mediated by the TβR-I/TβR-II/Smad2/3/4 signaling cascade, but TβR-V is not required for this TGF-β activity [3]. CHO-K1/vector, CHO-K1/mLRPI, CHO-K1/mLRPII, CHO-K1/mLRPIII, CHO-K1/mLRPIV, CHO-LRP-1$^-$/vector and CHO-LRP-1$^-$/LRP-1 cells were treated with 100 pM TGF-β_1 for 2 h. Northern blot analyses of PAI-1 and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) in these cells were performed. As shown in Fig. 6B, TGF-β_1 induced expression of PAI in all of these CHO cells expressing mLRPs (lanes 1–10), suggesting that TβR-I/TβR-II/Smad2/3/4 signaling is functional in CHO-K1 cells stably expressing mLRPs. It is also important to note that the TGF-β_1-induced expression of PAI-1 was greater in CHO-LRP-1$^-$/vector cells than in other CHO cells, including CHO-K1/vector and CHO-LRP-1$^-$/LRP-1 cells (lanes 13/14 versus lanes 1/2 or 11/12). This suggests that LRP-1/TβR-V may play a negative regulatory role in regulating TβR-I/TβR-II/Smad2/3/4 signaling.

4. Discussion

TGF-β is a potent growth inhibitor for many cell types [31–33]. The growth inhibitory activity of TGF-β has been implicated in many physiological and pathological processes. Understanding the mechanism by which TGF-β inhibits cell growth would be important to define the molecular basis of these biological processes. Accumulating evidence indicates that other signaling cascades, in addition to the classical TβR-I/TβR-II/Smad2/3/4 cascade, are involved in mediating the growth inhibitory response to TGF-β [34–40]. Recently, LRP-1/TβR-V has been shown to be required, in concert with other TGF-β receptor types, for mediating the growth inhibitory response to TGF-β_1 in epithelial cells and carcinoma cells [6]. There are several lines of evidence for this. (1) Reduced expression of LRP-1 by mutagenesis and selection with *Pseudomonas* exotoxin A attenuates the growth inhibitory response to TGF-β_1 in mink lung epithelial cells [6]. (2) Carcinoma cells expressing little or no TβR-V or LRP-1 do not exhibit the growth inhibitory response to TGF-β_1 [2,4,6,41,42]. (3) Stable transfection with LRP-1 cDNA restores sensitivity to TGF-β_1-induced growth inhibition in a human lung

carcinoma cell line (H1299 cells) [6]. Neither reduced expression of LRP-1 nor stable transfection with LRP-1 cDNA in these cells alters the expression of TβR-I, TβR-II and TβR-III in these cells. In addition, the TGF-β_1-induced transcriptional activation of PAI-1 is unaltered in these cells by the manipulation of LRP-1.

Since TGF-β_1 inhibits growth of many cell types, it would to be important to define the role of LRP-1 in the growth inhibitory response to TGF-β_1 in various cell types. Currently, there are two known cell systems available for defining the role of LRP-1 in TGF-β_1-induced growth inhibition using rescue approaches. These are mouse embryonic fibroblasts (MEF cells)/LRP-1-deficient mouse embryonic fibroblasts (PEA-13 cells) [22] and CHO-K1/CHO-LRP-1$^-$ cells [23]. We have attempted to perform rescue experiments by stably transfecting PEA-13 cells with LRP-1 cDNA. However, we have been unable to generate stable clones of transfected PEA-13 cells which produce LRP-1 at a level comparable to that in wild-type cells (MEF cells). There are at least two reasons for this: (1) MEF cells are fibroblasts that normally express $\geq$3-fold higher levels of LRP-1 than other cell types, e.g. epithelial cells [6], and (2) the size of LRP-1 cDNA is $\sim$15 kb and the LRP-1 transgene appears to be unstable in transfected cells [27]. On the other hand, we demonstrate here that CHO-LRP-1$^-$ cells stably transfected with LRP-1 cDNA in pCXN vector are relatively stable. They express a level of the LRP-1 transgene product comparable to that of LRP-1 in wild-type CHO cells (CHO-K1 cells), and should be suitable for defining the role of LRP-1 in the growth inhibitory response to TGF-β_1. However, we are unable to fully restore the growth inhibitory response to TGF-β in CHO-LRP-1$^-$ cells by stable transfection with LRP-1 cDNA to make them comparable to their parent CHO-K1 cells. In CHO-LRP-1$^-$/LRP-1 cells, TGF-β_1 inhibits DNA synthesis and cell growth by $\sim$5% and $\sim$30%, respectively. These are less than those of TGF-β_1-induced DNA synthesis and cell growth inhibition ($\sim$30% and $\sim$60%, respectively) in wild-type CHO cells (CHO-K1 cells). Possibly, the CHO-LRP-1$^-$ cells, which were originally generated by mutagenesis and selection with *Pseudomonas* exotoxin A, have alterations in other cellular events in addition to the loss of LRP-1 expression. Although the stable transfection of CHO-LRP-1$^-$ cells with LRP-1 cDNA only partially rescues the growth inhibitory response to TGF-β_1, it is clearly distinct from the mitogenic response to TGF-β_1 in the parent cells (CHO-LRP-1$^-$ cells).

mLRPs have been used to demonstrate the functions of each domain in ligand binding and endocytosis [27,43]. We used the procedures developed by them to generate mLRP constructs for studying the effects of overexpression of these mLRPs on the growth inhibitory response to TGF-β_1 in CHO-K1 cells. Unexpectedly, all of these mLRPs were found to be capable of attenuating the growth inhibitory response to TGF-β_1 in transfected CHO-K1 cells. The molecular mechanisms by which these mLRPs attenuate the growth inhibitory response to TGF-β_1 are currently unknown. Since all of these mLRPs have the same light chain, which includes the transmembrane and cytoplasmic domains, the light chains of these mLRPs may mediate the dominant negative mutant function.

Acknowledgements: This work was supported by National Institutes of Health Grant CA 38808 and grants from the Academia Sinica and the National Science Council, Taiwan. We thank Drs. William S. Sly and Frank E. Johnson for critical review of the manuscript; Chun-Lin Chen for performing [^{125}I]α_2M* binding analysis and John McAlpin for typing the manuscript. We are very grateful to Drs. Guejun Bu and Dudley K. Strickland for kindly providing CHO-LRP-1$^-$ cells, anti-LRP-1 sera and RAP, and to Dr. Joachim Herz for kindly providing pGEX-KG-RAP, pcDNA3.1(-)neo vector and pcDNA3.1 (-)neo-LRP-1 plasmids and anti-LRP-1 serum.

References

[1] Massague, J. (1992) Cell 69, 1067–1070.
[2] O'Grady, P., Huang, S.S. and Huang, J.S. (1991) Biochem. Biophys. Res. Commun. 179, 378–385.
[3] O'Grady, P., Kuo, M.D., Baldassare, J.J., Huang, S.S. and Huang, J.S. (1991) J. Biol. Chem. 266, 8583–8589.
[4] Leal, S.M., Liu, Q., Huang, S.S. and Huang, J.S. (1997) J. Biol. Chem. 272, 20572–20576.
[5] Leal, S.M., Huang, S.S. and Huang, J.S. (1999) J. Biol. Chem. 274, 6711–6717.
[6] Huang, S.S., Ling, T.-Y., Tseng, W.-F., Huang, Y.-H., Tang, F.-M., Leal, S.M. and Huang, J.S. (2003) FASEB 17, 2068–2089.
[7] Herz, J. and Strickland, D.K. (2001) J. Clin. Invest. 108, 779–784.
[8] Krieger, M. and Herz, J. (1994) Annu. Rev. Biochem. 63, 601–637.
[9] Strickland, D.K., Gonias, S.L. and Argraves, W.S. (2002) Trends Endocrinol. Metab. 13, 66–74.
[10] Howell, B.W. and Herz, J. (2001) Curr. Opin. Neurobiol. 11, 74–81.
[11] Herz, J., Hamann, U., Rogne, S., Myklebost, O., Gausepohl, H. and Stanley, K.K. (1988) EMBO J. 7, 4119–4127.
[12] Li, Y., Marzolo, M.P., van Kerkhof, P., Strous, G.J. and Bu, G. (2000) J. Biol. Chem. 275, 17187–17194.
[13] Li, Y., van Kerkhof, P., Marzolo, M.P., Strous, G.J. and Bu, G. (2001) Mol. Cell. Biol. 21, 1185–1195.
[14] Boucher, P., Liu, P., Gotthardt, M., Hiesberger, T., Anderson, R.G. and Herz, J. (2002) J. Biol. Chem. 277, 15507–15513.
[15] Loukinova, E., Ranganathan, S., Kuznetsov, S., Gorlatova, N., Migliorini, N.M., Loukinov, D., Mikhailenko, I., Lawrence, D.A. and Strickland, D.K. (2002) J. Biol. Chem. 277, 15499–15506.
[16] Strickland, D.K., Ashcom, J.D., Williams, S., Burgess, W.H., Migliorini, M. and Argraves, W.S. (1990) J. Biol. Chem. 265, 17401–17404.
[17] Bacskai, B.J., Xia, M.Q., Strickland, D.K., Rebeck, G.W. and Hyman, B.T. (2000) Proc. Natl. Acad. Sci. USA 97, 11551–11556.
[18] Qiu, Z., Strickland, D.K., Hyman, B.T. and Rebeck, G.W. (2002) J. Biol. Chem. 277, 14458–14466.
[19] Asplin, I.R., Misra, U.K., Gawdi, G., Gonzalez-Gronow, M. and Pizzo, S.V. (2000) Arch. Biochem. Biophys. 383, 135–141.
[20] Misra, U.K., Gawdi, G. and Pizzo, S.V. (1999) Arch. Biochem. Biophys. 372, 238–247.
[21] Lutz, C., Nimpf, J., Jenny, M., Boecklinger, K., Enzinger, C., Utermann, G., Baier-Bitterlich, G. and Baier, G. (2002) J. Biol. Chem. 277, 43143–43151.
[22] Willnow, T.E. and Herz, J. (1994) J. Cell Sci. 107, 719–726.
[23] Fitzgerald, D.J., Fryling, C.M., Zanovsky, A., Saelinger, C.B., Kounnas, M., Winkles, J.A., Strickland, D.K. and Leppla, S. (1995) J. Cell Biol. 129, 1533–1541.
[24] Herz, J., Goldstein, J.L., Strickland, D.K., Ho, Y.K. and Brown, M.S. (1991) J. Biol. Chem. 266, 21232–21238.
[25] Huang, S.S., O'Grady, P. and Huang, J.S. (1988) J. Biol. Chem. 263, 1535–1541.
[26] Liu, Q., Ling, T.Y., Shieh, H.S., Johnson, F.E., Huang, J.S. and Huang, S.S. (2001) J. Biol. Chem. 276, 46212–46218.
[27] Obermoeller-McCormick, L.M., Li, Y., Osaka, H., FitzGerald, D.J., Schwartz, A.L. and Bu, G. (2001) J. Cell Sci. 114, 899–908.
[28] Sells, M.A., Li, J. and Chernoff, J. (1995) BioTechniques 19, 72–76.
[29] Matsunami, K., Miyagawa, S., Nakai, R., Murase, A. and Shirakura, R. (2001) Clin. Exp. Immunol. 126, 165–172.
[30] Weaver, A.M., McCabe, M., Kim, I., Allietta, M.M. and Gonias, S.L. (1996) J. Biol. Chem. 271, 24894–24900.
[31] Roberts, A.B. and Sporn, M.B. (1990) in: Handbook of Experimental Pharmacology: Peptide Growth Factors andTheir Re-

ceptors (Sporn, M.B. and Roberts, A.B., Eds.), pp. 419–472, Springer-Verlag, New York.
[32] Massague, J. (1990) Annu. Rev. Cell Biol. 6, 597–641.
[33] Roberts, A.B. (1998) Miner. Electrolyte Metab. 24, 111–119.
[34] Taipale, J. and Keski-Oja, J. (1996) J. Biol. Chem. 272, 20572–20576.
[35] Hocevar, B.A. and Howe, P.H. (1998) Miner. Electrolyte Metab. 24, 131–135.
[36] Heldin, N.E., Bergstrom, D., Hermansson, A., Bergenstrahle, A., Nakao, A., Westermark, B. and ten Dijke, P. (1999) Mol. Cell. Endocrinol. 153, 79–90.
[37] Petritsch, C., Beug, H., Balmain, A. and Oft, M. (2000) Genes Dev. 14, 3093–3101.
[38] Fink, S.P., Swinler, S.E., Lutterbaugh, J.D., Massagué, J., Thiagalingam, S., Kinzler, K.W., Vogelstein, B., Wilson, J.K. and Markowitz, S. (2001) Cancer Res. 61, 256–260.
[39] Moustakas, A., Pardali, K., Gaal, A. and Heldin, C.H. (2002) Immunol. Lett. 82, 85–91.
[40] Liu, Q., Huang, S.S. and Huang, J.S. (1997) J. Biol. Chem. 272, 18891–18895.
[41] Gonias, S.L., LaMarre, J., Crookston, K.P., Webb, D.J., Wolf, B.B., Lopes, M.B., Moses, H.L. and Hayes, M.A. (1994) Ann. NY Acad. Sci. 737, 273–290.
[42] Li, Y., Wood, N., Parsons, P.G., Yellowlees, D. and Donnelly, P.K. (1997) Cancer Lett. 111, 199–205.
[43] Willow, T.E., Orth, K. and Herz, J. (1994) J. Biol. Chem. 269, 15827–15832.