Mutational analysis of the FXNPXY motif within LDL receptor-related protein 1 (LRP1) reveals the functional importance of the tyrosine residues in cell growth regulation and signal transduction

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LRP1 [LDLR (low-density lipoprotein receptor)-related protein 1]-null CHO cells (Chinese-hamster ovary cells) (13-5-1 cells) exhibited accelerated cell growth and severe tumour progression after they were xenografted into nude mice. Reconstitution of LRP1 expression in these cells, either with the full-length protein or with a minireceptor, reduced growth rate as well as suppressed tumour development. We tested the role of the tyrosine residue in the FXNPXY motif within the LRP1 cytoplasmic domain in signal transduction and cell growth inhibition by site-specific mutagenesis. The LRP1 minireceptors harbouring Tyr63 to alanine or Tyr63 to phenylalanine substitution had diametrically opposite effects on cell growth, cell morphology and tumour development in mice. The Y63F-expressing cells showed suppressed cell growth and tumour development, which were associated with decreased β-catenin and cadherin concentrations in the cells. On the other hand, the Y63A-expressing cells lacked inhibition on cell growth and tumour development, which were associated with hyperactivation of ERKs (extracellular-signal-regulated kinases), FAK (focal adhesion kinase) and cyclin D1 in the cells. The mutant Y63A minireceptor also exhibited reduced capacity in binding to the Dab2 (disabled 2) adaptor protein. In addition, the Y63A mutant showed increased caveolar localization, and cells expressing Y63A had altered caveolae architecture. However, tyrosine to alanine substitution at the other NPXY motif had no effect on cell growth or tumorigenesis. These results suggest that the FXNPXY motif of LRP1 not only governs cellular localization of the receptor but also exerts multiple functional effects on signalling pathways involved in cell growth regulation.

Key words: cell growth inhibition, extracellular-signal-regulated kinase (ERK), FXNPXY motif, low-density lipoprotein receptor-related protein 1 (LRP1), mutational analysis, tumorigenesis.

INTRODUCTION

LRP1 [LDLR (low-density lipoprotein receptor)-related protein 1] is a large (4525 amino acids) type I membrane protein composed of an extracellular α-chain (515 kDa) and a transmembrane β-chain (85 kDa) in the form of a non-covalently linked heterodimer [1]. The extracellular α-chain is composed of three modular structural repeats, namely class A ligand-binding (complement-type) repeats, epidermal growth factor precursor-type repeats and the β-propeller repeats [2,3]. The class A ligand-binding repeats in the α-chain are present in four clusters (designated I, II, III and IV), of which cluster II and cluster IV possess the ability to bind various structurally and functionally unrelated ligands [4]. The cytoplasmic tail of the β-chain of LRP1 contains NPXY and FXNPXY motifs, which resemble those required for receptor-mediated endocytosis in other LDLR family members [5,6]. In LRP1, the FXNPXY motif binds to endocytic adaptor proteins, including AP2 (adaptor protein 2), ARH (autosomal recessive hypercholesterolaemia) and Dab2 (disabled 2) [5,7–9], and mediates endocytosis through clathrin-coated pits.

In addition to its endocytic function, LRP1 has been increasingly recognized to play a role in cell growth regulation through signal transduction (for a review, see [10]). Thus many protein factors previously identified as ligands for LRP1-mediated endocytosis, such as urokinase plasminogen activator, plasminogen activator inhibitor 1, MMPs (matrix metalloproteinases), ApoE (apolipoprotein E), lactoferrin and TGF-β (transforming growth factor-β), also play roles in regulating cell migration, contraction and adhesion. Often, binding of these ligands to their respective cognate receptors requires LRP1 as a coreceptor in the same cell. In smooth-muscle cells, LRP1 may act as a co-receptor with PDGF (platelet-derived growth factor) receptor in mediating the PDGF-triggered signalling pathway [11]. Mice harbouring tissue-specific knockout of LRP1 in smooth-muscle cells developed severe atherosclerosis in the LDLR−/− background and exhibited profound up-regulation of PDGF receptor in the LRP1-deficient cells [11]. Similar coreceptor functions have been suggested for LRP1 in the Wnt signalling mediated through the Frizzled/LRP5/6 system [12] and in the TGF-β signalling cascade [13].

The endocytosis and signalling events of an endocytic receptor can be functionally interconnected [5]. The FXNPXY motif of LRP1 not only binds to endocytic APs but also serves as a binding site for signal transduction adaptor proteins, such as

Abbreviations used: AP2, adaptor protein 2; ApoE, apolipoprotein E; ARH, autosomal recessive hypercholesterolaemia; CHO cell, Chinese-hamster ovary cell; Dab2, disabled 2; EMT, endothelial mesenchymal transdifferentiation; ERK, extracellular-signal-regulated kinase; FAK, focal adhesion kinase; FBS, fetal bovine serum; Gb2, growth-factor-receptor-bound protein 2; GSK3, glycogen synthase kinase 3; HA, haemagglutinin; H/E, haematoyxlin and eosin; HEK-293 cells, human embryonic kidney cells; LDLR, low-density lipoprotein receptor; LRP1, LDLR-related protein 1; MAPK, mitogen-activated protein kinase; MEF, murine embryonic fibroblast; MEK, MAPK/ERK kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; PDGF, platelet-derived growth factor; PKC, protein kinase C; PKD, protein kinase D; RAP, receptor-associated protein; SOS, Son of Sevenless; TGF, transforming growth factor; TjR, TGF-β receptor.

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Dab1, SH3 domain (Src homology 3 domain) homology protein and insulin receptor substrate [6,8,14,15]. Thus it is of interest to determine if the FXNXPY motif within the LRP1 cytoplasmic domain plays a dual role in receptor-mediated endocytosis and in signal transduction. In the present study, we tested the role of tyrosine residues of the two NPYX motifs within LRP1 in signal transduction and cell growth using site-specific mutagenesis. Our results showed that replacement of tyrosine with alanine or phenylalanine at the FXNXPY motif of LRP1 had opposite effects on cell growth inhibition and suggested the involvement of this motif in multiple signal transduction pathways.

**EXPERIMENTAL**

**Construction and expression of LRP1 minireceptors**

The expression plasmid encoding the full-length human LRP1 (pcDNA3.1-hLRP1) (a gift from Dr Dudley K. Strickland) was used as a template to generate the minireceptor LRP1-IV that contained the cluster IV ligand-binding repeats. Two cDNA fragments were PCR-amplified with the Pfu Turbo polymerase; they encoded (from 5' to 3') LRP1 signal peptide, N-terminal five amino acids (AIDAP) of the mature LRP1 (GenBank accession no. X13916), HA (haemagglutinin) epitope (YPYDVPDYA), a three-amino-acid [GAP (Gly-Ala-Pro)] linker and the cluster IV ligand-binding repeats. Primer pair used to amplify the XhoI site, the LRP1 signal peptide sequence and the HA tag—AscI restriction site were 5'-TAATACGACTCACTATAGGG (forward) and 5'-GGGGCCGCAGCTTAATATGCACCAGCACGCTGCCACACTCAGTG (reverse). The primer pair used to amplify the cluster IV ligand-binding repeats and the AscI and NotI restriction sites were 5'-GGCGGGCTCCCACTGTTCC-ACGGCTAGCGAG (forward) and 5'-AAAAAGAAAAACGGCGCAGCTCTTGTGAAAG (reverse). The two amplified cDNA fragments were inserted into the pGEM-T vector, sequenced and subcloned into pcDNA3.1 at the multiple cloning sites XhoI and NotI. The resulting construct was designated LRP1-IVwt (or IVwt). The Tyr29 to alanine, Tyr63 to alanine or Tyr63 to phenylalanine substitution at the two NPXY motifs was introduced using the QuikChange® mutagenesis approach (Stratagene, La Jolla, CA, U.S.A.) to create Y29A, Y29A and Y63F variants (numbering starts at the first amino acid immediately adjacent to the transmembrane domain) [16,17].

The sequences of the mutagenesis primers for Y29A were: 5'-GATTGGACACCCACCCCGAGATGACGAGGC (forward) and 5'-GCCCTTGTACATCTTCCGCGTGGTTTCT-ACAT (reverse), primers for Y63A were: 5'-CTTCACCAACCCCGCTGAGCGCAG (forward) and 5'-CATGAGTGTGGCCGGTGGATG (reverse), and primers for Y63F were 5'-CTCTACCAACCCCGCTGAGCGCAG (forward) and 5'-CATGAGTGTGGCCGGTGGATG (reverse). The LRP1-IV minireceptors were stably introduced into the two NPYX motifs as a template to generate the minireceptor LRP1-IV that contained the cluster IV ligand-binding repeats. Two cDNA

**Cell migration assay**

Cells were cultured in serum-free media for 16 h prior to seeding at 5 × 10⁵ cells/ml (300 µl) into a Boyden chamber with 8.0 µm pore membranes (Chemicon, Billerica, MA, U.S.A.) that were coated with fibronectin or collagen I [21]. Control membranes were coated with BSA. The membranes were washed with serum-free F12 medium containing 2 mM CaCl₂ and 2 mM MgCl₂. Cell migration was allowed to proceed for 4 h with fibronectin-coated membranes or for 5 h with collagen I-coated membranes at 37°C. Both the upper and lower chambers contained serum-free medium. At the end of the incubation period, non-migrating cells were removed from the upper surface of the membranes with cotton swabs. The membranes were fixed and stained with 0.1% (w/v) Crystal Violet. The dye was eluted with 10% (v/v) acetic acid, and the absorbance (D) at 595 nm was determined.

**Tumour xenografts**

Female Balb/c nude mice (3–4 weeks of age) were purchased from Charles River and housed under specific pathogen-free facilities approved by the Canadian Association for Accreditation of Laboratories Animal Care. Cells expressing LRP1-IV minireceptors were grown to subconfluence, briefly trypsinized with 0.25% (w/v) trypsin in EDTA, washed with serum-free medium and resuspended in PBS. A total of 2.5 × 10⁶ cells in 150 µl of PBS were injected subcutaneously into the lower-dorsal region of the mice. Tumour development was measured in two dimensions (length and width) with a digital calliper, and tumour volume was calculated using the formula: (π/6) × width² × length [19]. The mice with tumour appearance were killed at day 16 post-injection, and tumours were excised and fixed in 10% (v/v) H₂O₂ in PBS. The specimens were blocked with 5% (v/v) H₂O₂ for 30 min, and then they were deparaffinized with xylene, dehydrated in alcohol and embedded in paraffin. Mice that showed no tumour appearance after 32 days were considered negative.

**Immunohistology of tumour**

Five micrometre slices of paraffin-embedded tumour samples were deparaffinized with xylene, dehydrated in alcohol and rehydrated in PBS. Endogenous peroxidase was blocked with 3% (w/v) H₂O₂ in PBS. The specimens were blocked with 5% (w/v) BSA in PBS and incubated overnight at 4°C with an anti-LRP1 β-chain antibody. After 1 h incubation at room temperature with peroxidase-conjugated secondary anti-rabbit antibody, positive reaction was detected by exposure to stable 3,3’-diaminobenzidine tetrahydrochloride (Sigma, Oakville, ON, Canada). Slips were...
counterstained with Shandon Instant Hematoxylin. Sections stained for immunoperoxidase or H/E (haematoxylin and eosin) were examined using the Nikon Eclipse 80i microscope equipped with a QIMAGING MicroPublisher 5.0 RTV camera. Digital images were captured using Image Pro Plus version 5 software.

**Immunocytochemistry**

For detection of LRP1 cell surface presentation, cells were grown on fibronectin-coated coverslips for 24 h, washed three times with ice-cold PBS, and fixed on ice with 4% (w/v) paraformaldehyde for 30 min. The fixed cells were washed three times with 100 mM glycine, blocked with 1% BSA for 1 h, incubated with a monoclonal anti-HA antibody and subsequently with goat anti-mouse IgG conjugated with Alexa Fluor® 488 (Invitrogen, Burlington, ON, Canada). For detection of intracellular F-actin, the fixed cells were treated with Triton X-100 [0.1% (v/v) in PBS] for 5 min at room temperature (20°C). The permeabilized cells were incubated in blocking solution [10% (w/v) FBS (fetal bovine serum) in PBS] for 20 min, and stained with Alexa Fluor® 660 phalloidin (2.5%, w/v) (Invitrogen) in blocking solution for 1 h at room temperature. For caveolin-1 staining, the fixed and permeabilized cells were blocked with FBS (10% in PBS) for 1 h, and incubated with anti-caveolin-1 polyclonal antibody and subsequently with goat anti-mouse IgG conjugated with Alexa Fluor® 599 (Invitrogen). The coverslips were mounted on to a glass slip using SlowFade Light AntiFade (Invitrogen), and the images were captured using OLYMPUS Fluoview 1000 and the MellesGriot laser scanning confocal imaging system.

**Immunoblot analysis**

Cells were collected into ice-cold PBS containing 2 mM NaF/2 mM Na3VO4 and lysed with a lysis buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1% (v/v) Triton X-100, 2 mM MgCl2, 2 mM EGTA, 2 mM EDTA, 2 mM glycerophosphate, 2 mM NaF, 2 mM Na3VO4, and a cocktail of protease inhibitors. The cell lysate was separated by PAGE in the presence of SDS (SDS/PAGE) and the resolved proteins were transferred on to 0.2 µm pore nitrocellulose membranes (Bio-Rad, Hercules, CA, U.S.A.). The membranes were incubated with appropriate primary antibodies for 16 h at 4°C and secondary antibody for 2 h at room temperature. Primary antibodies used were: anti-pY 4G10 (Upstate, Billerica, MA, U.S.A.), polyclonal anti-LRP1 β-chain [22], monoclonal anti-HA (BabCO, Richmond, CA, U.S.A.), anti-FAK (focal adhesion kinase), anti-phospho-FAK (Tyr992), anti-ERK (extracellular signal-regulated kinase) [p44/42 MAPK (mitogen-activated protein kinase)], anti-phospho-ERK (Thr202/Tyr204) anti-cadherin, anti-cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), anti-caveolin-1 (C13630) and anti-p96 (Da62) (BD Transduction Laboratories, Mississauga, ON, Canada), goat anti-human/rat β-catenin (R&D Systems, Minneapolis, MN, U.S.A.) and monoclonal anti-HSP60 (Sigma). The immunocomplexes were visualized by the BM chemiluminescence method (Roche).

**Co-immunoprecipitation**

Cell lysates were precleared with preimmune IgG and Protein A beads. The lysate was adjusted to a final concentration of 20% (w/v) sucrose and incubated with primary antibody for 1 h and Protein A beads (eBioscience, San Diego, CA, U.S.A.) for another 1 h at 4°C. The beads were washed five times with the lysis buffer. The co-immunoprecipitated proteins were eluted with reducing SDS sample buffer [25 mM Tris/HCl, pH 8.0, 150 mM NaCl, 10% (w/v) SDS, 50 mM DTT (dithiothreitol) and 40 mM 2-mercaptoethanol], separated by SDS/PAGE (8% gel) and detected using TrueBlot (eBioscience). Anti-Dab2 polyclonal antibody used in the co-immunoprecipitation experiments was a gift from Dr Xiang-Xi Xu (Department of Medical Oncology, Fox Chase Cancer Center, Philadelphia, PA, U.S.A.) or obtained from Santa Cruz Biotechnology (sc-13982).

**Kinase expression profiling**

Kinase expression was profiled by Western-blot analysis, a service provided by Kinexus (Vancouver, BC, Canada).

**Other assays**

RAP (receptor-associated protein)-specific binding to cell surface LRP1 was performed using 125I-RAP (5.0 nM) at 4°C in the absence or presence of 50-fold excess of unlabelled RAP as described previously [23,24]. Isolation of membrane rafts was performed using the detergent (Triton X-100) solubilization method as described previously [22]. Cholesterol oxidase (0.5 unit/ml) treatment was conducted at 37°C for 60 min as described previously [25].

**RESULTS**

**Restoration of LRP1 expression in LRP1-null CHO cells reduced cell growth and tumorigenesis**

The LRP1-null CHO cells, designated 13-5-1, have been used as a model system for LRP1 structure–function relationship studies [13,23,26,27]. We noted that the LRP1-null cells exhibited accelerated growth rate as compared with parental CHO-K1 cells (results not shown). It has been shown previously by several clinical studies that LRP1 expression is either aberrant or entirely lost in several cancers [28–32]. Since these studies suggest a potential role of LRP1 in regulating cell growth, we thus first tested the effect of restoration of LRP1 expression on cell growth using two LRP1 constructs, namely the full-length human LRP1 (hLRP) and a minireceptor termed LRP1-IV (IVwt) that contains cluster IV of the ligand-binding repeats (see the top two lines in Figure 1A). The LRP1-IV minireceptor has also been used for LRP1 structure–function studies and is shown to possess normal endocytic functions for many LRPI ligands [17,22,33]. Stable transfection of 13-5-1 with either hLRP or IVwt resulted in normal expression and post-translational cleavage into the α- and β-chains, and the level of transgene expression was comparable with that in 3T3-L1 and HEK-293 cells (human embryonic kidney cells) (bottom panels in Figure 1A). Noticeably, F-actin staining showed that morphological changes occurred from fibroblastoid spindle shape in vector-transfected control cells to squamous shape in hLRP- or IVwt-expressing cells (Figure 1B). Moreover, expression of hLRP or IVwt resulted in markedly reduced stress fibre formation. Similar morphological changes in LRP1-null cells after LRP transfection have been observed previously [13]. Stable expression of hLRP and IVwt resulted in decreased cell growth by 40 and 30% respectively as compared with cells transfected with vector alone (Figure 1C). Likewise, anchorage-independent colony formation in soft-agar was decreased by 50 and 40% respectively (Figure 1D). Expression of hLRP or IVwt also markedly inhibited fibronectin-induced cell migration as compared with vector-transfected control cells (Figure 1E). [These cells showed limited migration on type I collagen-coated substratum (Figure 1E).] These results provide the first indication that restoration of LRP1 expression in the LRP1-null cells, either with the full-length protein or a minireceptor, resulted in cell growth inhibition.
It has been observed previously that expression of full-length LRP1 in LRP1-null cells attenuated tumorigenicity in nude mice (J.S. Huang, personal communication). In the present studies, we confirmed that subcutaneous xenografting of LRP1-null cells indeed resulted in severe tumour development in nude mice (top panel in Figure 2A). All mice started to develop tumours at day 7 after injection, and the tumour volume progressed at the rate of ~40 mm³/day to day 16 (Figure 2B). Expression of IVwt, as was observed for the full-length LRP1, resulted in significantly reduced tumour development (bottom panel in Figure 2A). Only half of the mice injected with IVwt cells developed tumours, and the tumours grew at a low rate of ~10 mm³/day (Figure 2B). At day 16, the tumour volume in mice injected with vector-transfected control cells was 751.6 ± 161.6 mm³, whereas, in mice injected with IVwt cells, it was 233.0 ± 116.6 mm³ (P < 0.05) (compare top and bottom panels of Figure 2A). Mice injected with untransfected parental LRP-null (13-5-1) cells exhibited the same tumour growth rate as those injected with vector-transfected control cells (results not shown). These in vivo results, consistent with the reported growth inhibition by LRP1 expression [13,34], indicate that the LRP1-IV minireceptor possesses functional elements essential for the inhibition of cell growth and tumour development.

Tyr63 to alanine substitution abolished the ability of LRP1-IV to inhibit cell growth

The cell growth inhibition by IVwt expression suggests that the C-terminal portion of LRP1 contains the information essential for this function. We postulated that the cell growth inhibition function might be linked to one of the two NPxY motifs residing within the cytoplasmic domain of LRP1. To test this hypothesis, we performed site-specific mutagenesis at tyrosine residues (i.e. the putative phosphorylation site) to introduce Tyr²⁹ to alanine, Tyr⁶³ to alanine and Tyr⁶³ to phenylalanine substitutions (top lines in Figure 3A). These constructs were stably transfected into 13-5-1 cells, and multiple clones that expressed comparable levels of LRP1-IV variants were selected (representative clones are shown in the bottom panels of Figure 3A). Immunostaining with anti-LRP1 β-chain antibody showed clear signals of the transgene product in tumour specimens obtained from mice injected with IVwt cells (Figure 2C). Histological analysis with H/E staining did not reveal significant differences in the phenotypes (e.g. area of necrosis) of tumour cells between samples obtained from the two groups of mice (results not shown). These in vivo results, consistent with the reported growth inhibition by LRP1 expression [13,34], indicate that the LRP1-IV minireceptor possesses functional elements essential for the inhibition of cell growth and tumour development.
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possibility that abnormal cell growth was attributable to clonal
observed in multiple clones of Y63A cells, ruling out the
cell growth and anchorage-independent colony formation were
higher than IVwt, Y29A or Y63F cells (Figure 3F). Enhanced
was no noticeable difference in their turnover rate among these
variants during chase. Results from these kinetic studies ruled out
the possibility that replacing the amino acid tyrosine with alanine
variants during chase. Results from these kinetic studies ruled out
the possibility that replacing the amino acid tyrosine with alanine
phenylalanine residues causes protein misfolding.

However, unlike that of IVwt, expression of Y63A in the
LRP1-null cells failed to exert an inhibitory effect on cell growth
or anchorage-independent colony formation. Thus Y63A cells
exhibited a growth rate that was similar to the vector-transfected
cell colonies on soft-agar was similar to vector control, but was ∼35 %
higher than IVwt and Y63F cells, respectively (Figure 3F). Likewise, the formation of Y63A cell
colonies on soft-agar was similar to vector control, but was ∼35 %
higher than IVwt, Y29A or Y63F cells (Figure 3F). Enhanced
cell growth and anchorage-independent colony formation were
observed in multiple clones of Y63A cells, ruling out the
possibility that abnormal cell growth was attributable to clonal
variation. These results suggest that Tyr63 to alanine mutation, but
not Tyr29 to phenylalanine or Tyr63 to alanine mutation, severely
compromises LRP1’s ability to inhibit cell growth.

The loss of growth inhibition by Tyr63 to alanine mutation
was further exemplified by in vivo analysis of the progression
of tumorigenesis in xenografted nude mice. Stably transfected
cell lines expressing comparable levels of the respective LRP1
minireceptors were injected into the mice at two different
doses. At low dose (0.5 × 10⁶ cells) the incidence of tumour
development was 86 % (4 out of 6 mice) for vector transfected
cell (LRP-null), 14 % (1 out of 7) for IVwt, 14 % (1 out of
7) for Y29A, 17 % (1 out of 6) for Y63F and 33 % (2 out of 5)
for Y63A. At high dose (2.5 × 10⁶ cells), all of the mice (8 out
of 8) injected with LRP-null cells developed tumours within 5–
7 days post-injection. The incidence of tumour development was
reduced to 36 % (5 out of 9) in IVwt-injected mice (P < 0.001
compared with LRP-null). The incidence of tumour development
in Y63A-injected mice was 89 % (8 out of 9), almost as high
as in mice injected with LRP-null (0.05 < P < 0.1 compared with
LRP-null). Mice injected with Y29A or Y63F cells also exhibited
low frequency in tumour development (78 % for Y29A and 75 %
for Y63F) as compared with LRP-null (P < 0.01). In addition to
differences in the frequency of tumour development, there were
also marked differences in the rate of tumour growth (Figure 4B).
Mice injected with Y63A cells exhibited fast tumour growth rate (∼30 mm³/day) that was similar to that observed in mice
injected with LRP1-null (i.e. vector-injected control) (top panel
in Figure 4A). In contrast, mice injected with Y63F or Y29A
cells displayed lowered tumour growth rates (∼10 mm³/day) that
resembled the growth rate observed in mice injected with IVwt
cells (middle and bottom panels in Figure 4A). Immunostaining
of Y63A cells present in the tumour specimens showed more
condensed Y63A signals in the cells as compared with that in
Y29A, Y63F, (Figure 3C) and IVwt cells (Figure 2C). Together,
mutational analysis of the FXNPXY motifs with cell culture and
nude mice models suggests a functional domain that is critical for
receptor function in cell growth inhibition. Moreover, retention
of normal cell growth inhibition in the Y63F mutant suggests that
the presence of the aromatic tyrosine side chain, not a potential
phosphorylation site, is essential for FXNPXY function.

Hyperactivation of the MEK (MAPK/ERK kinase)/ERK pathway by
Y63A expression

To gain insights into mechanisms responsible for the link between
the FXNPXY motif and receptor function in cell growth, we

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Figure 2  In vivo tumorigenesis assay

(A) Subcutaneous tumour developed in nude mice at day 16 post-injection. Individual tumour volume was measured at the indicated days after injection of vector-transfected control cells or IVwt
cells (2.5 × 10⁶ cells per injection). Average volume of tumours (means ± S.E.M.) is shown at the indicated days post injection. Between two groups of mice, *P < 0.05 at days 11, 14 and 16. (B) The rates of tumour growth between the two groups of mice. *P < 0.05 (IVwt compared with vector control). (C) Immunohistochemical analysis of IVwt expression with tumour biopsy samples 16 days after injection.
profiled the expression and activation pattern of protein kinases. We contrasted the IVwt and Y63A cells that showed inhibition and lack-of-inhibition respectively of cell growth. Of 75 kinases examined, 11 showed increases and two showed decreases (by $\geq 40\%$) in Y63A cells as compared with IVwt cells. The kinases involved in the MAPK pathway [ERK1, MEK2 and JNKs (c-Jun N-terminal kinases)] were mostly up-regulated in Y63A cells [Figure 5A(a)], whereas, the kinases involved in the Wnt signalling pathway [e.g. protein kinase CK2 and GSK3 (glycogen synthase kinase 3)] were down-regulated [Figure 5A(b)]. Down-regulation of protein kinase CK2 and GSK3 probably resulted in increased stability of $\beta$-catenin in Y63A cells (see below). Kinases involved in the PKB (protein kinase B) pathway and other receptor tyrosine kinases [e.g. FAK, Lyn (a Src family tyrosine kinase), Csk (C-terminal Src kinase) and Src] were variably altered by Y63A expression [Figure 5A(c)]. Kinases involved in the PKC (protein kinase C)/PKD (protein kinase D) pathway, particularly PKC$\xi$ and CaMKIId (Ca$^{2+}$/calmodulin-dependent protein kinase 1D), were up-regulated [Figure 5A(d)]. These kinase profiling results provide the first indication that expression of IVwt, not the Y63A mutant, may suppress activation of several major signalling pathways in the cells.

Since aberrant activation of the ERK pathway is involved in accelerated cells growth and transformation, we examined further the effect of FXNPXY mutations on ERK activation. Constitutive ERK activation was observed in Y63A and vector-transfected control cells as compared with that in IVwt and Y63F cells when serum was present in the media (Figure 5B), indicative of hyperactivation of ERK in the absence of functional LRP1. In another experiment where the cells were serum-deprived for 18 h and then cultured in the media $\pm$ FBS for 30 min, robust ERK phosphorylation in response to serum treatment occurred in IVwt and Y63A cells but not in Y63F cells (top panel in Figure 5C). Under the same conditions, phosphorylation of cyclin D1, which is subjected to regulation of ERK and Wnt/$\beta$-catenin signalling pathways [35], was slightly activated in Y63A cells under serum-deprived conditions (as compared with IVwt cells) and was further activated by serum supplementation. However, in Y63F cells, cyclin D1 phosphorylation was increased under serum-free conditions (as compared with IVwt cells) yet its response to serum was entirely absent (middle panel in Figure 5C). The lack of an effect of serum treatment on cyclin D1 phosphorylation in Y63F cells corroborates further that cell growth inhibition by LRP1 expression is related to blunted ERK activation.

To identify factors responsible for enhanced anchorage-independent colony formation and constitutive activation of ERK in Y63A cells, we determined the effect of treatment with the MEK inhibitor PD98059. PD98059 treatment completely inhibited colony formation (Figure 5D) and diminished ERK phosphorylation in Y63A cells (inset in Figure 5D). Colony formation of Y63A-expressing cells was only partially inhibited by the PI3K (phosphoinositide 3-kinase) inhibitor LY290042 (Figure 5D). Treating the cells with the p38 MAPK inhibitor SB202190 had no effect on ERK phosphorylation (results not shown). Thus upstream regulation of hyperactivation of ERK in Y63A cells was probably mediated by MEK. These results suggest
Mutational analysis of the FXNPXY motif within LRP1

Figure 4  In vivo tumorigenesis assay

(A) Subcutaneous tumour developed in nude mice. The experiment was performed as in Figure 2, except that cells expressing different LRP1-IV variants were used. Average volume of tumours (means ± S.E.M.) is shown at the indicated days post injection. * P < 0.05 (Y29A compared with Y63A at day 16); ** P < 0.01 (Y63F compared with Y63A at day 16). (B) Rates of tumour growth. Values for vector-transfected control cells and IVwt cells shown in Figure 2(B) are included here for comparison purposes. The rate of tumour growth for Y63A cells is not statistically different from vector-transfected control cells. * P < 0.05 (IVwt and Y29A compared with vector control); ** P < 0.01 (Y63F compared with vector control). (C) Immunohistochemical analysis of expression of LRP1-IV variants within tumour biopsy samples at day 16 post-injection.

that the loss of cell growth inhibition in Y63A cells was associated with constitutive activation of the MEK → ERK cascade.

Opposing effects of Y63A and Y63F on β-catenin levels

It has been shown that deregulation of both the Wnt/β-catenin and ERK pathways can lead to cell transformation [36,37]. We thus examined the level of β-catenin and its phosphorylation status in cells expressing LRP1-IV variants. The level of β-catenin was slightly increased in Y63A cells compared with that in IVwt cells. On the other hand, β-catenin levels were markedly decreased in multiple stable Y63F clones and this was accompanied by decreased cadherin and FAK (as well as phosphorylated FAK) (data derived from a representative Y63F stable clone are shown in Figure 6A). The decreased β-catenin in Y63F cells was not merely attributable to enhanced proteasome-mediated degradation, because treating cells with the proteasome inhibitor MG-132 (the proteasome inhibitor carbobenzyoxyl-L-leucyl-L-leucyl-leucinal) did not equalize the β-catenin levels between Y63F cells and other cells (top panel in Figure 6B). The level of phosphorylated Ser37/Thr41 β-catenin, the substrate of proteasomal degradation, was similar among all cells in the presence of MG-132 (bottom panel in Figure 6B). Immunocytochemistry analysis showed prominent staining of β-catenin at the cell–cell boundaries in all cells except in Y63F cells where plasma membrane localization of β-catenin was markedly decreased (Figure 6C). The diminished association of β-catenin with plasma membrane might be the result of lowered cadherin levels in these cells. A striking contrast in morphology and stress fibre formation was observed between Y63A and Y63F cells (Figure 6D), the former resembled vector-transfected control cells and the latter resembled IVwt cells (see Figure 1B). These results suggest that expression of Y63A and Y63F variants exerted opposing effects on plasma-membrane-associated proteins that are important for cytoskeleton arrangement, focal adhesion and extracellular matrix formation.

To search for potential link(s) between mutations within the LRP1 FXNPXY motif and the profound changes in cell morphology and in ERK activation, we determined interaction of LRP1 β-chain and various adaptor proteins by co-immunoprecipitation. Among various adaptor proteins examined (including AP2, ARH and Dab2), only Dab2 showed a drastic difference in binding between Y63A and Y63F. Immunoprecipitation of the cell lysates with anti-Dab2, and subsequent immunoblot analysis for LRP1 β-chain and Dab2 showed markedly reduced interaction between Y63A mutant and Dab2 (Figure 6E, lane 8), whereas interaction of Y29A (lane 6) and Y63F (lane 10) with Dab2 was normal as compared with IVwt (lane 4). Notably, the level of Dab2 was decreased in Y63F cells (lane 10).

The Y63A-expressing cells had altered caveolae architecture

Finally, we determined the effect of mutations at the YXNPXY motif on the receptor protein localization to lipid rafts or caveolae. Lipid rafts are membrane microdomains rich in cholesterol and signalling molecules [38]. Caveolin-1 is a membrane-bound protein that specifies the lipid rafts termed caveolae. We have shown previously that LRP1 localization to lipid rafts is sensitive to insulin and PDGF treatment in differentiated 3T3-L1 adipocytes [22]. Examination of caveolin-1 distribution showed a dramatic increase in caveolin-1 association with lipid rafts in
cells that exhibited loss of growth inhibition (i.e. vector control and Y63A cells) (the four left-hand side panels in Figure 7A). Increased caveolin-1 association with lipid rafts was observed in Y63A cells even after treatment with cholesterol oxidase (four right-hand side panels in Figure 7A), suggesting that some of the lipid rafts were not present on the cell surface. There were no significant changes in the total cellular caveolin-1 concentrations between different cell lines as determined by immunoblot analysis of total cell lysates (results not shown). Immunocytochemical analysis showed prominent caveolin-1 signals present on the surface of Y63A cells (Figure 7B). A small proportion of Y63A (<1% of total) was reproducibly observed in lipid rafts (Figure 7C). These results suggest that the architecture of lipid rafts or caveolae, a platform of signal transduction, is altered by expression of the Y63A mutant.

**DISCUSSION**

Lack of functional LRP1 is associated with hyperactivation of ERK

The current site-specific mutagenesis analysis attempts to unravel functional roles of the tyrosine residues within the two NPXY motifs of human LRP1 in cell growth regulation and signal transduction. We have identified the distal FXNPXY motif as a functional domain for cell growth inhibition and ERK activation. Cells deficient in LRP1, either because of the lack of LRP1 expression (in LRP1-null cells) or the lack of functional FXNPXY motif (in Y63A cells), displayed aberrantly constitutive activation of MEK→ERK→cyclin D1 (Figures 5B and 5C). Although mechanisms by which deficiency in normal LRP1 expression can lead to hyperactivation of the ERK pathway remain to be defined, we found that restoring LRP1 expression could effectively suppress cell growth and blunt ERK activation, suggesting strongly that LRP1 indeed plays a role in antiproliferation. It was reported previously that LRP1-deficient MEFs (murine embryonic fibroblasts) had elevated phospho-ERK when cells were serum-starved for 18 h, and restoration of LRP1 expression in these cells suppressed ERK hyperactivation [39]. The level of ERK phosphorylation, however, was equivalent between LRP1-deficient and normal MEFs when serum was present in the media [39]. In the present study, we found that ERK hyperactivation occurred in vector-transfected LRP1-null cells and Y63A cells even when the media were supplemented with serum, and the expression of IVwt or Y63F suppressed ERK phosphorylation (Figure 5B). Differences in ERK phosphorylation between Y63A and Y63F cells were also observed when the cells were first deprived and then re-supplemented with serum (Figure 5C). Thus our results, in agreement with previous observations with the LRP1-deficient MEF cells, suggest that LRP1 plays a role in signal transduction through regulating the ERK pathways. Recently, several studies have shown that ERK activation through LRP1 occurs in other cell types as well. For example, the LRP1...
Mutational analysis of the FXNPXY motif within LRP1

Figure 6 Opposing effects of Y63A and Y63F on β-catenin and other plasma-membrane-associated proteins

(A) Immunoblot analysis of β-catenin, cadherin, phospho-FAK (anti-phospho-FAK (Tyr397)) and FAK. (B) Immunoblot analysis of β-catenin and phospho-β-catenin in cells treated with (+) or without (−) MG-132. (C) Immunocytochemical analysis of β-catenin (green). Nucleus was stained with DAPI (4′,6-diamidino-2-phenylindole) (blue). (D) Cells were stained with Alexa Fluor® 660 phalloidin to visualize F-actin. (E) Co-immunoprecipitation of Dab2 and LRP1 β-chain. An anti-Dab2 polyclonal antibody was used to pull down complexes and the precipitates were probed for LRP1 β-chain (top panel) and Dab2 p96 (bottom panel) respectively.

Figure 7 Altered caveolar architecture in Y63A-expressing cells

(A) Cell homogenates were subjected to lipid raft fractionation using the detergent method as described in the Experimental section. After sucrose-density-gradient ultracentrifugation, the fractionated samples were probed for caveolin-1 (left-hand side panels). Samples derived from cells treated with cholesterol oxidase (CO) prior to fractionation are shown in the right-hand side panels. (B) Immunocytochemical analysis of caveolin-1 using a polyclonal anti-caveolin-1 antibody. (C) Immunoblots of LRP1 β-chain. IB, immunoblot.

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ligand lactoferrin-induced mitogenesis of rat osteoblastic cells requires functional LRPI and is associated with increased ERK phosphorylation [40]. Also, TGF-β-mediated differentiation of rat kidney fibroblasts can be potentiayed by connective tissue growth factor, another LRPI ligand, that can activate ERK in a process associated with transient phosphorylation of tyrosine within the LRPI β-chain [41]. Our mutational analysis of the Tyr to alanine or Tyr to phenylalanine substitution showing differential responses in ERK activation provides new evidence implicating the involvement of the FXNPXY motif of LRPI in ERK activation.

Signalling function of LRPI is specifically linked to the FXNPXY motif

The present studies also provide new evidence linking the FXNPXY motif of LRPI to cell growth inhibition (Figures 3E and 3F) and tumour progression (Figure 4). The opposing effects of Y63A and Y63F mutations on cell growth regulation are accompanied by differential alterations in the levels of β-catenin, cadherin and FAK (Figure 6A), in addition to altered ERK activation. Two pieces of experimental evidence suggest that involvement of the FXNPXY motif is not attributable to tyrosine phosphorylation. First, in the cultured CHO cells, we did not detect phosphorylation of the Tyr residue under any culture conditions tested (H. Zhang, unpublished work). Secondly, the fact the Y63F mutant, but not the Y63A mutant, retained the normal LRPI function in suppressing cell growth and tumour development suggests that the presence of an aromatic side chain is probably more important than phosphorylation in conferring LRPI action. The lack of an effect of Tyr to alanine substitution on the LRPI cell growth inhibition indicates that there is no functional redundancy between the NPXY29 and FXNPXY63 motifs. Moreover, the loss of cell growth inhibition by Tyr to alanine substitution observed in the present study was unlikely to be attributable to abnormally high or low expression of the mutant, because the effect was observed with multiple clones of which the level of Y63A expression was comparable with other variants tested. Thus our work suggests a specific cell growth inhibition function associated with the FXNPXY63 motif. Recently, an in vivo transgenic mice work in which the NPXY63 motif of the mouse LRPI was replaced (using knock-in technology) with amino acids AAVA63 showed that Tyr to alanine substitution caused no observable phenotypic changes in the resulting animals [42]. However, mutations at the NPXY29 motif (NPXY-to-AATA) resulted in destruction of liver tissue between E16.5 (embryonic day 16.5) and E18.5 [42]. The reason for the lack of a manifestation of the NPXY29 motif in cell growth inhibition in vivo remains to be explained. It is possible that selective interaction between NPXY29 and adaptor proteins for cytokine signallning is organ-specific and also developmental stage-specific [42], which may not be revealed in ovariun-originated CHO cells in vitro. Therefore the selective interaction of adaptor proteins with NPXY motifs in a tissue- and time-specific manner may play important roles in diverse functions of LRPI.

Pleiotropic effects of FXNPXY mutations on signalling pathways

The two NPXY motifs in the cytoplasmic domain of LRPI β-chain have been shown to interact with a variety of adaptor and scaffold proteins, and some of the adaptor proteins are potentially involved in mitogenic signal transduction in addition to mediating endocytosis [14,43]. However, the biological significance of LRPI binding to these adaptor proteins remains largely unclear. We have discovered in the present study that mutation within the FXNPXY motif has a profound effect on the level of Dab2 (e.g. in the case of Y63F) and its interaction with LRPI β-chain (e.g. in the case of Y63A mutation) (Figure 6E). Dab2 is involved in regulation of endocytosis of LDLR family members such as LDLR, ApoER2 (ApoE receptor 2) and megalin [44], through interacting with the FXNPXY motif irrespective of the phosphorylation status of the tyrosine residue [6,15]. Dab2 is a known tumour suppressor; its expression in human ovarian carcinoma cells suppresses cell growth rate and tumour formation in nude mice [45,46], whereas homozygous knockout of Dab2 results in early embryonic lethality [45]. Factors that are known to regulate ERK activation include Grb2 (growth-factor-receptor-bound protein 2) and SOS (Son of Sevenless), and the ERK activation by Grb2 and SOS can be attenuated by Dab2 through its interacting with and sequestering Grb2 [44,47]. In the present mutagenesis studies, hyperactivation of ERK in cells deficient in LRPI (either because LRPI-null or Y63A mutation) was closely associated with reduced interaction between LRPI and Dab2 (Figures 5B and 6E). It remains to be determined if the decreased LRPI-Dab2 interaction in LRPI-null and Y63A cells is responsible for hyperactivation of ERK.

LRPI has been recognized as a member of the TβR (TGF-β receptor) family and it can mediate TGF-β-induced growth inhibition independent of other TβRs [13,34]. During the early stage of tumorigenesis, TGF-β acts as a tumour suppressor principally through its ability to cause growth arrest and apoptosis, whereas at late stage of tumorigenesis, TGF-β promotes tumour progression through its ability to induce morphological changes termed EMT (endothelial mesenchymal transdifferentiation) [48]. The process of EMT is characterized by loss of cell–cell interaction and acquisition of fibroblastic morphology. The present experiments with cells expressing the full-length LRPI or the minireceptor IVwnt (Figure 1B) show that cytoskeleton reorganization (visualized by F-actin) occurred in these cells that exhibited cell growth inhibition. Thus lack of LRPI expression results in cell transdifferentiation under non-stimulated conditions (i.e. in the absence of exogenous TGF-β). Our results are in agreement with previously reported morphological changes (from fibroblastoid spindle shape to wild-type squamous shape) observed in 13-5-1 cells transfected with the LRPI minigene [13]. In mouse mammary gland epithelial cells, Dab2 is shown to play a role in TGF-β-induced EMT, probably through activation of integrin [49].

Dab2 also plays a role in the Wnt/β-catenin signalling [50]. Notably, concomitant changes in β-catenin protein levels and ERK phosphorylation were observed in the Y63A and Y63F cells in the absence of extracellular stimuli (Figures 5B and 6A). The Wnt/β-catenin pathway is known to play a role in ERK activation during growth stimulation [36,37]. Defective Wnt signalling is associated with cancer, osteoporosis and other diseases [51]. Members of the LDLR family, LRP5/6, act as coreceptors of Frizzled in the canonical Wnt/β-catenin signalling [52]. On the other hand, LRPI has been shown to interact with Frizzled and negatively regulate the signalling process by sequestering Frizzled [12]. The Y63A-expressing cells that lost cell growth inhibition displayed up-regulated intracellular β-catenin, and the increased β-catenin was not merely a result of decreased proteasomal degradation (Figure 6B). These results provide additional evidence that expression of LRPI indeed negatively regulates the Wnt/β-catenin signalling pathway. Thus ERK hyperactivation observed in the Y63A-expressing cells under non-stimulated conditions is probably achieved through interplay of multiple signalling molecules including Dab2 and β-catenin as suggested by other studies [36,53].

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