Identification and Characterization of the Acidic pH Binding Sites for Growth Regulatory Ligands of Low Density Lipoprotein Receptor-related Protein-1*

Thai-Yen Ling*, Chun-Lin Chen*, Yen-Hua Huang†, I-Hua Liu*, Shuan Shian Huang‡, and Jung San Huang††

From the †Institute of Biomedical Sciences, Academia Sinica, Taipei 115, Taiwan, the ‡Department of Biochemistry and Molecular Biology, St. Louis University School of Medicine, St. Louis, Missouri 63104, and the ¶Department of Biochemistry, Taipei Medical University, Taipei 110, Taiwan.

The type V TGF-β receptor (TβR-V) plays an important role in growth inhibition by IGFBP-3 and TGF-β in responsive cells. Unexpectedly, TβR-V was recently found to be identical to the LRP-1/αvM receptor; this has disclosed previously unreported growth regulatory functions of LRP-1. Here we demonstrate that, in addition to expressing LRP-1, all cells examined exhibit low affinity but high density acidic pH binding sites for LRP-1 growth regulatory ligands (TGF-β1, IGFBP-3, and αvM). These sites, like LRP-1, are sensitive to receptor-associated protein and calcium depletion but, unlike LRP-1, are also sensitive to chondroitin sulfate and heparin and capable of directly binding ligands, which do not bind to LRP-1. Annexin VI has been identified as a major membrane-associated protein capable of directly binding αvM at acidic pH. This is evidenced by: 1) structural and Western blot analyses of the protein purified from bovine liver plasma membranes by αvM affinity chromatography at acidic pH, and 2) dot blot analysis of the interaction of annexin VI and 125I-αvM. Cell surface annexin VI is involved in 125I-TGF-β1 and 125I-αvM binding to the acidic pH binding sites and 125I-αvM binding to LRP-1 at neutral pH as demonstrated by the sensitivity of cells to pretreatment with anti-annexin VI IgG. Cell surface annexin VI is also capable of mediating internalization and degradation of cell surface-bound 125I-TGF-β1 and 125I-αvM at pH 6 and of forming ternary complexes with 125I-αvM and LRP-1 at neutral pH as demonstrated by co-immunoprecipitation. Trifluoperazine and fluphenazine, which inhibit ligand binding to the acidic pH binding sites, block degradation after internalization of cell surface-bound 125I-TGF-β1 or 125I-αvM. These results suggest that cell surface annexin VI may function as an acidic pH binding site or receptor and may also function as a co-receptor with LRP-1 at neutral pH.

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† To whom correspondence may be addressed: Dept. of Biochemistry and Molecular Biology, St. Louis University School of Medicine, 1402 South Grand Blvd., St. Louis, MO 63104. Tel.: 314-977-9251; Fax: 314-977-9205; E-mail: huangjs@slu.edu.

‡ To whom correspondence may be addressed: Dept. of Biochemistry and Molecular Biology, St. Louis University School of Medicine, 1402 South Grand Blvd., St. Louis, MO 63104. Tel.: 314-977-9250; Fax: 314-977-9205; E-mail: huangjs@slu.edu.
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Indication of IGFBP-3, TGF-β3, and αM—IGFBP-3 or TGF-β3 (5 μg) was iodinated with 2 μCi of Na125I using chloramine T according to the procedure of Leal et al. (4, 5) and O’Grady et al. (2), respectively. The specific radioactivities of 125I-labeled IGFBP-3 (125I-IGFBP-3) and 125I-labeled TGF-β3 (125I-TGF-β3) were 1 × 106 cpm/ng and 1 × 107 cpm/ng, respectively. Indication of αM (100 μg) was done as described previously (23–25). The specific radioactivity of 125I-labeled αM (125I-αM) was 2 × 108 cpm/ng. 125I-TGF-β3 or 125I-αM was mixed with unlabeled TGF-β3 or αM to yield a specific radioactivity of 2 × 105 cpm/ng in specific experiments.

Specific Binding of 125I-IGFBP-3, 125I-TGF-β3, and 125IαM to Cells—Mv1Lu, MEF, and PEA-13 cells were plated at a cell density of 8 × 104 cells/well in 48-well clustered dishes and grown at 37°C in DMEM/50 mM HEPES, pH 7.4 containing 10% fetal calf serum. The cells were then washed and incubated with 6 nm 125I-IGFBP-3, 1 nm 125I-TGF-β3, or 125I-αM in the presence or absence of EGTA (tetrasodium salt) or BAPTA (5 mM), GST-RAP (15 μg/ml), or 200-fold excess of unlabeled TGF-β3 or αM in DMEM/50 mM HEPES/acetate at pH 4.0, 5.0, 6.0, 7.4 (or 7.0), and 8.0, all containing BSA (1 mg/ml). GST-RAP or 200-fold excess of unlabeled TGF-β3 or αM was used to estimate nonspecific binding. After 2.5 h at 0°C, the specific binding of 125I-IGFBP-3, 125I-TGF-β3, or 125I-αM was determined. BAPTA and the tetrasodium salt (but not the free acid form) of EGTA appeared to function well as chelators of Ca2+ at acidic pH (26).

The experiments were performed in quadruplicate.

Immunoprecipitation of Cell Surface-bound 125I-TGF-β3 or 125I-αM—Cells (8 × 104 cells/well) in 48-well clustered dishes were incubated with 125I-TGF-β3 (100 μCi) or 125I-αM (2 μCi) with or without 10 μM trifluoperazine, fluphenazine, or promethazine in the presence and absence of 200-fold excess of unlabeled TGF-β3 or αM (to estimate nonspecific binding) in DMEM/25 mM HEPES, pH 7.4 containing BSA (1 mg/ml). After 2 h at 0°C, the cells were washed and incubated with DMEM/25 mM HEPES, pH 7.4 containing BSA (1 mg/ml) with or without 10 μM trifluoperazine, fluphenazine, or promethazine. After 1 h at 37°C, the medium was collected and precipitated with 10% trichloroacetic acid. The trichloroacetic acid-soluble radioactive material in the medium represented the cellular degradation products of 125I-TGF-β3 or 125I-αM. The cells were then treated with trypsin (5 μg/ml) in DMEM/25 mM HEPES activated by methylation with 10% formaldehyde at 0°C and centrifuged. The radioactivity in the supernatant and cell pellets represented cell surface-bound and internalized 125I-TGF-β3 or 125I-αM, respectively. The experiments were performed in quadruplicate.

Immunoprecipitation of Cell Surface-bound 125I-αM—MEF and PEA-13 cells (1 × 106 cells/well) in 24-well clustered dishes were incubated with 1 nm 125I-αM in the presence and absence of 200-fold excess of unlabeled αM in DMEM/25 mM HEPES, pH 7.4 containing BSA (1 mg/ml). After 2 h at 0°C, the cells were lysed with 50 μM HEPES/50 mM HEPES buffer, pH 7.4 containing 0.1% Triton X-100, 0.15 mM NaCl, and 2 mM Ca2+, and the cell lysates were immunoprecipitated with an antibody to annexin VI IgG and then washed, fixed, and incubated with anti-rabbit IgG-fluorescein isothiocyanate conjugate (1:50 dilution) at room temperature for 1.5 h. After washing, the cells were incubated with anti-rabbit IgG-fluorescein isothiocyanate conjugate (1.5 dilution) at room temperature for 1.5 h and then washed twice with ice-cold phosphate-buffered saline prior to visualization with a confocal fluorescent microscope. Cell Surface Localization of Annexin VI—Cells grown on coverslips in DMEM/25 mM HEPES, pH 7.4 containing 10% fetal calf serum were fixed with 3.7% formaldehyde in DMEM/25 mM HEPES, pH 7.4 containing 10% calf serum, washed, fixed with 3.7% formaldehyde in DMEM/50 mM HEPES, pH 7.4 containing 10% calf serum, and then washed twice with ice-cold phosphate-buffered saline prior to visualization with a confocal fluorescent microscope.
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LRP-1 Ligands Exhibit High Capacity Binding to Cells at Acidic pH—LRP-1 is a 600-kDa type I membrane glycoprotein which has been shown to bind more than 35 ligands (10–12). These ligands have different structures and functions but share two receptor binding properties: 1) Ca\(^{2+}\) dependence and 2) inhibition by RAP. Since IGFBP-3 and TGF-β1 are the newly identified ligands for LRP-1, we determined whether they share these two receptor binding properties with αM in Mv1Lu cells. Mv1Lu cells are a standard model cell system for investigating TGF-β1 and IGFBP-3 receptors and activities (4, 5, 27–29). These cells were incubated with 6 nM 125I-IGFBP-3, 1 nM 125I-TGF-β1, or 10 nM 125I-αM in the presence or absence of GST-RAP (15 μg/ml), EGTA or BAPTA (5 μM), or 200-fold excess of unlabeled IGFBP-3, TGF-β1 or αM at pH 4, 5, 6, 7, 4 (or 7.0), and 8.0. After 2.5 h at 0 °C, the specific binding of 125I-IGFBP-3, 125I-TGF-β1, and 125I-αM was determined. GST-RAP is a fusion protein of glutathione S-transferase and RAP, which inhibits binding of all known ligands to LRP-1 (9, 10, 19, 30, 31). The tetrasodium salt (but not the free acid form) of EGTA functions well as a chelator of Ca\(^{2+}\) at acidic pH. BAPTA is a Ca\(^{2+}\) chelator independent of pH (26). As shown in Fig. 1, A–C, 125I-IGFBP-3, 125I-TGF-β1, and 125I-αM bound to Mv1Lu cells in a pH-dependent manner. The specific binding (GST-RAP-sensitive) of 125I-IGFBP-3, 125I-TGF-β1, and 125I-αM exhibited a maximum at pH 5. The specific binding (GST-RAP-sensitive) of 125I-IGFBP-3, 125I-TGF-β1, and 125I-αM at pH 7.4 was much less than at pH 5 (Fig. 1, A–C). The pH profiles of the EDTA- or BAPTA-sensitive binding for these radioactive ligands were similar to those of the GST-RAP sensitive binding (data not shown). The apparent Kd values for binding of IGFBP-3, 125I-TGF-β1, and 125I-αM to TjR-V/LRP-1 at pH 7.4 are known to be 6 nM, 50–400 pM, and 75 pM, respectively (7, 8, 32). These results suggest that Mv1Lu cells may possess low affinity, high density binding sites (which are GST-RAP- and Cu\(^{2+}\) depletion-sensitive) for 125I-IGFBP-3, 125I-TGF-β1, and 125I-αM with acidic pH optima. This suggestion is supported by Scatchard plot analysis of 125I-αM binding to Mv1Lu cells at pH 5. As shown in Fig. 2A, 125I-αM bound to Mv1Lu cells in a concentration-dependent manner at pH 5 with a saturating concentration of 120 nM. Scatchard plot analysis of the binding data revealed a single class of low affinity binding sites with an apparent Kd of 54 nM and 1.5 × 10\(^6\) sites/cell (Fig. 2B). The Kd values of the low affinity acidic pH binding sites for 125I-IGFBP-3 and 125I-TGF-β1 were not determined. However, it is very possible that the Kd values of the low affinity acidic pH binding sites for 125I-IGFBP-3 and 125I-TGF-β1 are similar to the apparent Kd of the acidic pH binding sites for 125I-αM. We therefore focused on characterizing 125I-TGF-β1 and 125I-αM binding to the acidic pH binding sites in all of the following experiments.

Since binding of LRP-1 ligands (125I-IGFBP-3, 125I-TGF-β1, and 125I-αM) to the acidic pH binding sites requires the presence of Ca\(^{2+}\) and is sensitive to GST-RAP, we suspected that LRP-1 itself might mediate binding. LRP-1 is known to have ligand binding activity with a neutral pH optimum. To exclude this possibility, we performed 125I-TGF-β1 or 125I-αM binding at a varying pH using mouse embryonic fibroblasts (MEF) and LRP-1-deficient mouse embryonic fibroblasts (PEA-13 cells). As shown in Fig. 1, D and E, binding in both MEF and PEA-13 cells was maximal at pH 5, suggesting that such binding is mediated by a protein(s) other than LRP-1. These results are also consistent with the notion that LRP-1 does not have significant ligand binding activity at acidic pH and that LRP-1 unloads bound ligands in endosomes (due to acidic pH-induced ligand dissociation) following internalization.

The Acidic pH Binding Sites in Cells Have Broad Ligand Specificity—αM is a plasma protease inhibitor that inhibits all four classes of proteases. αM is cleaved by the protease in the bait region (which is close to the thioester bond in the αM three-dimensional structure) and undergoes conformational changes, resulting in trapping of the protease (21–25). The protease-activated αM is cleaved αM, or αM can be mimicked by methylamine-treated αM since methylamine also cleaves the same thioester bond, thus inducing the same conformational changes in αM (21–25). LRP-1 has been shown to bind αM and native αM with different affinities (Kd 40–75 pM and 2 nM, respectively) (10–14). To see if, like LRP-1, the acidic pH binding sites have different affinities for αM and native αM, we determined the effects of increasing concentrations of unlabeled αM and native αM on 125I-αM binding at pH 5.5 in MEF cells. As shown in Fig. 3A, increasing concentrations of αM and native αM correspondingly inhibited 125I-αM binding to the acidic pH binding sites with IC50 values of 50 nM and 150 nM, respectively. The IC50 of unlabeled αM appeared to be similar to the apparent Kd for 125I-αM binding to the acidic pH binding sites in Mv1Lu, MEF, and PEA-13 cells as determined by Scatchard plot analysis (Fig. 2 and Table I). We also determined the effects of increasing concentrations of unlabeled αM and native αM on 125I-αM binding to PEA-13 cells which are known to be deficient in LRP-1. As shown in Fig. 3B, unlabeled αM and native αM also inhibited 125I-αM binding to the acidic pH binding sites in a concentration-dependent manner with IC50 values of 120 nM and > 400 nM, respectively. These IC50 values were higher than those found in wild-type cells (MEF cells). This result suggests that in PEA-13 cells, the absence of LRP-1 may decrease the binding affinity of native αM or αM for acidic pH binding sites. Alternatively, LRP-1 may collaborate with the acidic pH binding sites for ligand interactions at acidic pH.

The ligands of endocytic receptors such as transferrin, lactoferrin (a LRP-1 ligand) and LDL have also been shown to exhibit acidic pH binding in various cell types (33–35), but they have not been well characterized. To determine whether the
acidic pH binding sites for \( \alpha_2 \text{M}^* \) are also responsible for binding transferrin, lactoferrin and apoE at acidic pH in cells, we first examined the effects of these proteins on \( \text{I}^{125} \)-\( \alpha_2 \text{M}^* \) binding to Mv1Lu, MEF, and PEA-13 cells. Cells were incubated with 2 nM \( \text{I}^{125} \)-\( \alpha_2 \text{M}^* \) in the presence and absence of 100 \( \mu \text{M} \) transferrin, lactoferrin, or apoE at pH 5.5 or pH 7.4 (for comparison). After 2.5 h at 0°C, the specific binding of \( \text{I}^{125} \)-\( \alpha_2 \text{M}^* \) to cells was determined. At 100 \( \mu \text{M} \), all of these proteins completely blocked the specific binding (at pH 5.5) of \( \text{I}^{125} \)-\( \alpha_2 \text{M}^* \) in Mv1Lu, MEF, and PEA-13 cells (data not shown). In contrast, none of these proteins had a significant effect on \( \text{I}^{125} \)-\( \alpha_2 \text{M}^* \) binding (at pH 7.4) to Mv1Lu and MEF cells, which is mediated by LRP-1 (data not shown). Lactoferrin and \( \alpha_2 \text{M}^* \) bind to distinct sites of LRP-1 and do not compete with each other for binding to LRP-1 (10, 12). These results suggest that transferrin, lactoferrin and apoE may bind to the same acidic pH binding sites as \( \alpha_2 \text{M}^* \) does. Alternatively, the acidic pH binding sites for these molecules may be different but overlapping. To further define the ligand specificity of the acidic pH binding sites, the effects of various concentrations of transferrin, lactoferrin, \( \gamma \)-globulin, \textit{Pseudomonas} exotoxin A (also a ligand of LRP-1) (36), or LDL on \( \text{I}^{125} \)-\( \alpha_2 \text{M}^* \) binding (at pH 5 or 6) to Mv1Lu cells were determined. As shown in Fig. 4, A and B, increasing concentrations of lactoferrin, transferrin, and LDL correspondingly inhibited \( \text{I}^{125} \)-\( \alpha_2 \text{M}^* \) binding to the acidic pH binding sites in Mv1Lu cells with \( \text{IC}_{50} \) values of 0.05 \( \mu \text{M} \) (pH 5), 0.5 \( \mu \text{M} \) (pH 5), and 5 \( \mu \text{g/ml} \) (pH 6), respectively. In contrast, \( \gamma \)-globulin and \textit{Pseudomonas} exotoxin at 0.5 \( \mu \text{M} \) did not effectively inhibit \( \text{I}^{125} \)-\( \alpha_2 \text{M}^* \) binding to the acidic pH binding sites (data not shown). These results indicate that the acidic binding sites are capable of binding ligands, which do not bind to LRP-1 (e.g. transferrin).

Cell Surface Annexin VI Is Involved in the Acidic pH Binding of \( \text{I}^{125} \)-\( \alpha_2 \text{M}^* \) and \( \text{I}^{125} \)-TGF-\( \beta_1 \) in Cells—The acidic pH binding sites on the cell surface identified herein may participate in the process of ligand endocytosis and degradation. They may be co-internalized with LRP-1/\( \alpha_2 \text{M}^* \) receptors and possibly other unidentified receptors with ligand binding activities with neutral pH optima) and become activated in endosomes which have acidic luminal pH (pH 5.5–6.5) through acidic pH-induced conformational changes. They may also be present in endosomes where they function as intracellular cargo transporters, which target ligands for lysosomal degradation. To identify the membrane-associated protein(s) responsible for mediating the acidic pH binding, we decided to purify this protein(s) from Triton X-100 extracts of bovine plasma membranes by \( \alpha_2 \text{M}^* \)-Sepharose affinity column chromatography at pH 5 or 6.
plasma membranes were used as the starting material because they are rich in endocytic receptors such as LRP-1 (9, 10). If these acidic binding sites have collaborative interactions with the endocytic receptors in vivo, they should be abundant in tissues (e.g., liver) and cells that are rich in endocytic receptors. The Triton X-100 extracts (pH 5 or 6) containing 4 mM CaCl₂ of bovine liver plasma membranes were subjected to ²H₂M*-Sepharose 4B affinity column chromatography at pH 5 or 6. After extensive washing with HEPES/acetate buffer at pH 5 or 6 containing 0.1% Triton X-100 and 4 mM CaCl₂, the column was eluted with HEPES/acetate buffer at pH 5 or 6 containing 10 mM EDTA and 0.1% Triton X-100 and the eluted fractions analyzed by silver staining. As shown in Fig. 5A, a 68-kDa protein was found in the EDTA eluent fractions (from pH 6 affinity column chromatography), as demonstrated by 7.5% SDS-PAGE under non-reducing conditions and silver staining. MALDI-TOF analysis of the tryptic digests of this 68-kDa protein revealed that the protein was bovine annexin VI (data not shown). Western blot analysis of the 68-kDa protein also supported the conclusion that it was annexin VI (Fig. 5B, lane 2). Under the experimental conditions (affinity column chromatography at pH 6), a very small amount of LRP-1 was found in the EDTA eluent fractions. LRP-1 is known to have ligand binding activity with a neutral pH optimum (10–12). However, annexin VI appeared to be the major protein in the EDTA eluents of ²H₂M*-Sepharose 4B affinity column chromatography at pH 5 (data not shown) or 6. These results suggest that bovine annexin VI is an ²H₂M*-binding protein. To further define the direct interaction of annexin VI with ²H₂M*, we performed dot blot analysis. Pure annexin I, II, III, IV, and VI (0.1 µg) were immobilized on nitrocellulose membranes. After blocking with BSA, the membranes were probed with ¹²⁵I⁻²H₂M* at pH 4, 5, 6, and 7 in the presence of 2 mM Ca²⁺. As shown in Fig. 6, ¹²⁵I⁻²H₂M* directly interacts with annexins in a pH-dependent manner. Binding of ¹²⁵I⁻²H₂M* to these annexins was maximum at pH 5. Quantitation of ¹²⁵I⁻²H₂M* bound to these annexins revealed that annexin VI bound more ¹²⁵I⁻²H₂M* than other annexins in the order of annexin VI > annexin IV > annexin III > annexin I > annexin II. Annexin V was also found to be as effective as annexin IV for binding ¹²⁵I⁻²H₂M* at pH 5 (data not shown). Binding of ¹²⁵I⁻²H₂M* to these annexins was abolished in the presence of RAP (100 µg/ml) or BAPTA (5 mM) (data not shown). These results suggest that annexin VI as well as other annexins are capable of directly binding ²H₂M* with an optimum pH of 5.0. They are also consistent with the contention that cell surface annexin VI is involved in the acidic pH binding of ²H₂M* in cells.

Annexin VI has recently been shown to be a putative cell surface receptor for chondroitin sulfate (37). It was also reported to be capable of binding heparin (38, 39). We, therefore, examined the effects of chondroitin sulfate and heparin on ¹²⁵I⁻²H₂M* binding to Mv1Lu cells at pH 5. As shown in Fig. 7A,
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Fig. 3. αM* exhibits higher affinity than native αM to the acidic pH binding sites in MEF (A) and PEA-13 (B) cells. MEF (A) and PEA-13 (B) cells were incubated with 2 nM 125I-αM* in the presence of increasing concentrations (as indicated) of unlabeled αM or native αM (αM) at pH 5.5 (DMEM/HEPES/acetate). After 2.5 h at 0°C, the cell-associated specific binding of 125I-αM* was determined. The 125I-αM* binding obtained in the absence of unlabeled αM and αM was taken as 100% binding. Each data point is the mean ± S.D. of quadruplicate determinations.

Table I

|         | Mv1Lu cells | MEF | PEA-13 cells* |
|---------|-------------|-----|-------------|
| Kd (nM)| 57 ± 6      | 67 ± 11 | 50          |
| Receptor number | 1.2 ± 0.3 x 10^6 | 1.4 ± 0.1 x 10^6 | 1 x 10^6  |

* Two independent analyses were performed. The data are represented as the average of duplicate determinations.

chondroitin sulfate A, B, and C were potent inhibitors of 125I-αM* binding at pH 5. Interestingly, chondroitin sulfate B and C were more potent than chondroitin sulfate A in inhibiting 125I-αM* binding to the acidic pH binding sites. In contrast, heparin at 1 μg/ml enhanced 125I-αM* binding to the acidic pH binding sites by 400% (Fig. 7B). At 10 μg/ml, heparin inhibited 125I-αM* binding to these cells by >80% (Fig. 7B). Since annexin VI is known to bind chondroitin sulfate and heparin, these results are consistent with the contention that cell surface annexin VI is involved in the acidic pH binding of 125I-αM*.

The molecular mechanism by which heparin at 1 μg/ml increases 125I-αM* binding to the acidic pH binding sites is unknown. Heparin at 1 μg/ml may increase 125I-αM* binding to the acidic pH binding sites by removal of inhibitor activity. Heparin has also been shown to increase and decrease binding of other ligands at low and high concentrations, respectively (40). At 10 μg/ml, chondroitin sulfate A, B, and C and heparin did not significantly affect 125I-αM* binding to LRP-1 at neutral pH in Mv1Lu cells (data not shown).

To prove that cell surface annexin VI is involved in the acidic pH binding of LRP-1 ligands, we examined the effect of anti-annexin VI IgG treatment at pH 6.4 and 7.4 (for subsequent binding assays at pH 6.4 and 7.4, respectively) on 125I-TGF-β1 binding to Mv1Lu cells or 125I-αM* binding to MEF and PEA-13 cells. The anti-annexin VI IgG was highly specific and did not react with other annexins as determined by Western blot analysis and Coomassie Blue staining (Fig. 8, A and B, respectively). Cells were treated with various concentrations of anti-annexin VI IgG or control IgG at pH 6.4 and 7.4 at 37°C for 2 h. The choice of pH 6.4 incubation (instead of pH 5.0 or 5.5) was to allow appropriate interaction of the antigen and antibody. The specific binding (at pH 6.4 and 7.4) of 125I-TGF-β1 binding to Mv1Lu cells or 125I-αM* was then determined. As shown in Fig. 9, increasing concentrations of anti-annexin VI IgG quantitatively blocked 125I-TGF-β1 binding to pH 6.4 in Mv1Lu cells (Fig. 9A), 125I-αM* binding to pH 6.4 in Mv1Lu, MEF, and PEA-13 cells (Fig. 9, B–D, respectively) and 125I-αM* binding at pH 7.4 in Mv1Lu and MEF cells (Fig. 9, E and F). Anti-annexin VI IgG at 25 μg/ml completely blocked the 125I-αM* binding at pH 6.4 in Mv1Lu, MEF, and...
IgG (B).

Western blot analysis using anti-annexin VI fraction (lane 1) were analyzed by Western blot analysis with 10 μl EDTA in HEPES/acetate buffer. The fractional volume was 1 ml. An aliquot of fractions (EDTA eluents) was subjected to 7.5% SDS-PAGE under non-reducing conditions and silver stained. The peak fraction (fraction 33) containing a 68-kDa protein (lane 2) and the concentrated column flow-through fraction (lane 1) were analyzed by Western blot analysis using anti-annexin VI IgG (B). The arrowhead indicates the location of annexin VI.

Fig. 5. Annexin VI binds to α, M*-Sepharose 4B in a calcium-dependent manner. The Triton X-100 extracts of bovine liver plasma membranes were applied onto a column of α, M*-Sepharose 4B (1.6 × 20 cm) in HEPES/acetate, pH 6.0, 0.15 M NaCl, 0.1% Triton X-100 (HEPES/acetate buffer) containing 4 mM Ca²⁺. After washing with HEPES/acetate buffer extensively, the column was eluted with 10 mM EDTA in HEPES/acetate buffer. The fractional volume was 1 ml. An aliquot of fractions (EDTA eluents) was subjected to 7.5% SDS-PAGE under non-reducing conditions and silver stained. M indicates the protein molecular mass standards (175, 62, 47, and 33 kDa). The peak fraction (fraction 33) containing a 68-kDa protein (lane 2) and the concentrated column flow-through fraction (lane 1) were analyzed by Western blot analysis using anti-annexin VI IgG (B). The arrowhead indicates the location of annexin VI.

Fig. 6. Annexin VI and other annexins directly interact with 125I-α, M*. Dot blot analysis was performed to analyze the direct interactions of pure annexin I, II, III, IV, and VI with 125I-α, M* at pH 4, 5, 6, and 7. Dots of annexins immobilized on nitrocellulose membranes were probed with 125I-α, M* as described in the text and analyzed by autoradiography. The relative intensities of 125I-α, M* bound to the annexins were quantitated by a PhosphorImager and estimated to be 1.0, 0.5, 0.4, 0.3, and 0.1 for annexin VI, annexin III, annexin II, annexin I, and annexin II, respectively.

Fig. 7. Chondroitin sulfate (A) and heparin (B) inhibit 125I-α, M* binding (at acidic pH) to Mv1Lu cells. Cells were incubated with 2 μM 125I-α, M* with or without 15 μg/ml GST-RAP (to estimate nonspecific binding) in the presence of various concentrations (as indicated) of chondroitin sulfate A, B, and C (A) or heparin (B). After 2 h at 4°C, the specific binding of 125I-α, M* was determined. The specific binding of 125I-α, M* obtained in the absence of chondroitin sulfate and heparin was taken as 100% binding. Each data point is the mean ± S.D. of quadruplicate determinations.

Mv1Lu, MEF, PEA-13, and Hep3B cells (Fig. 10, A, E, G, and C, respectively). Annexin VI has also been localized at the cell surface of other cell types (37, 38). To test the above possibilities, we performed co-immunoprecipitation of cell surface-bound 125I-α, M* (at pH 7.4) in MEF cells and PEA-13 cells using anti-annexin VI IgG. As shown in Fig. 11, anti-annexin...
VI IgG was capable of co-immunoprecipitating 125I-2M* (40% of LRP-1-bound 125I-2M*) in MEF cells (lane 1) but not in PEA-13 cells (lane 3). Since MEF and PEA-13 cells express comparable levels of annexin VI as determined by Western blot analysis (data not shown), this result indicates that LRP-1-bound 125I-2M* in MEF cells can be co-immunoprecipitated by anti-annexin VI IgG. It also suggests that cell surface annexin VI may form ternary complexes with 125I-2M* and LRP-1 and function as a co-receptor of LRP-1.

Cell Surface Annexin VI Is Involved in Ligand Binding, Internalization, and Degradation in Cells—Because PEA-13 cells are deficient in LRP-1 (20) and because the density of LRP-1 is nearly 100% occluded by annexin VI, the results suggest that annexin VI may be involved in ligand binding and degradation in cells. Western blot analysis of annexins using anti-annexin VI IgG (Fig. 8) shows that annexin VI is present in all cell lines tested. Treatment of cells with anti-annexin VI IgG prevents the binding of 125I-TGF-β1 or 125I-α2M* to Mv1Lu (A, B, E), MEF (C, F), and PEA-13 (D) cells at pH 6.4 (A–D) or pH 7.4 (E, F). Cells were treated with increasing concentrations (as indicated) of anti-annexin VI IgG or control IgG at pH 6.4 or pH 7.4 at 37°C for 2 h. The specific binding of 125I-TGF-β1 (0.1 nM) or 125I-α2M* (1 nM) in these cells treated with anti-annexin VI IgG or control IgG was then determined after incubation of cells with 125I-TGF-β1 (A) or 125I-α2M* (B–F) at pH 6.4 (A–D) or 7.4 (E, F) at 0°C for 2 h. The specific binding of 125I-TGF-β1 or 125I-α2M* at pH 6.4 or 7.4 in cells treated without anti-annexin VI IgG and control IgG was taken as 100% binding. Each data point is the average of triplicate determinations.
Acidic pH Binding Sites and Cell Surface Annexin VI

Specific Inhibitors Block Acidic pH Ligand Binding in Cells—Fluphenazine was previously shown to be an annexin VI binding compound as demonstrated by affinity column chromatography (41). Since fluphenazine and other phenothiazine-related compounds, which are weak bases, are capable of entering cells and accumulating at high concentration in intracellular acidic compartments (e.g. endosomes) (42, 43), it seemed possible that fluphenazine and similar compounds (e.g. trifluoperazine) may affect LRP ligand binding to annexin VI in the lumen of endosomes and prelysosomal compartments. To test this possibility, we examined the effects of several weak bases including trifluoperazine (a phenothiazine) (43), fluphenazine (another phenothiazine) (41), monodansylcadaverine (a transglutaminase inhibitor) (44), promethazine (another phenothiazine) (41), W-5 (a weak calmodulin antagonist) (46), W-7 (a potent calmodulin antagonist) (46), and verapamil (a calcium channel blocker) (47) on $^{125}$I-$\alpha_2$M$^*$ binding to MEF cells and PEA-13 cells at pH 5.5 and pH 7.4. Among these compounds, trifluoperazine and fluphenazine were found to be the most potent inhibitors of $^{125}$I-$\alpha_2$M$^*$ binding to MEF cells at pH 5.5 (Fig. 12A). Monodansylcadaverine and W-7 were less effective inhibitors. Promethazine, whose structure is homologous to trifluoperazine and fluphenazine, was not effective in blocking $^{125}$I-$\alpha_2$M$^*$ binding to cells at pH 5.5. Verapamil and W-5 (100 $\mu$M) were inactive in blocking $^{125}$I-$\alpha_2$M$^*$ binding to cells at pH 5.5 (data not shown). Trifluoperazine and fluphenazine inhibited $^{125}$I-$\alpha_2$M$^*$ binding in a concentration-dependent manner with $IC_{50}$ values of 65–75 $\mu$M at pH 5.5. Trifluoperazine and fluphenazine also appeared to be effective in inhibiting $^{125}$I-$\alpha_2$M$^*$ binding to LRP-1 at pH 7.4 (Fig. 12B). The $IC_{50}$ values of the trifluoperazine and fluphenazine were estimated to be 25–30 $\mu$M (Fig. 12B). Interestingly, promethazine was almost as effective as trifluoperazine and fluphenazine for inhibiting $^{125}$I-$\alpha_2$M$^*$ binding (at pH 7.4) to LRP-1 in MEF cells (Fig. 12B). These results suggest that trifluoperazine and fluphenazine are capable of blocking the binding of LRP ligands (e.g. $\alpha_2$M$^*$) to acidic pH binding sites or annexin VI and may be useful agents for defining the biological functions of the acidic
The results of Table II demonstrate the effect of trifluoperazine and fluphenazine on the degradation of cell surface-bound 125I-TGF-β1 binding to MEF cells. In the absence of 125I-TGF-β1 or 125I-α-M5+ in Mv1Lu cells, the specific binding of 125I-TGF-β1 or 125I-α-M5+ was determined. As shown in Table III, trifluoperazine at 10 μM inhibited specific binding at pH 5 of 125I-TGF-β1 in a concentration-dependent manner with an IC50 of 150 μM. This result suggests that trifluoperazine also blocks 125I-TGF-β1 binding to the acidic pH binding sites (e.g., annexin VI) effectively.

To determine the effect of trifluoperazine on 125I-TGF-β1 binding to the acidic pH binding sites, Mv1Lu cells were incubated with 100 pm 125I-TGF-β1 in the presence of various concentrations of trifluoperazine. After 2.5 h at 0 °C, the specific binding of 125I-TGF-β1 was determined. As shown in Fig. 12C, trifluoperazine inhibited specific binding at pH 5 of 125I-TGF-β1 in a concentration-dependent manner with an IC50 of 150 μM. This result suggests that trifluoperazine also blocks 125I-TGF-β1 binding to the acidic pH binding sites (e.g., annexin VI) effectively.

**Table II**

| 125I-α-M5+ | Cells (pH 6.0) | MEF       | Cells (pH 7.4) | MEF       |
|------------|----------------|-----------|----------------|-----------|
|            | PEA-13    | cpm/well % |                | cpm/well % |
| Cell surface-bound | 3,337 ± 79 (34) | 2,684 ± 70 (32) | 162 ± 23 (9) | 162 ± 23 (9) |
| Internalized | 3,926 ± 61 (40) | 3,635 ± 91 (44) | 1,046 ± 91 (56) | 1,046 ± 91 (56) |
| Degraded   | 2,552 ± 37 (26) | 1,971 ± 32 (24) | 660 ± 87 (35) | 660 ± 87 (35) |

**Fig. 12.** Trifluoperazine and fluphenazine inhibit 125I-α-M5+ binding to MEF cells at pH 5.5 and 7.4 (A, B) and 125I-TGF-β1 binding to Mv1Lu cells at pH 5 (C) in a concentration-dependent manner. MEF cells were incubated with 1 nM 125I-α-M5+ (A–C) or 100 pm 125I-TGF-β1 (C) in the presence of various concentrations (as indicated) of trifluoperazine (TFP), fluphenazine (FL), promethazine (PM), W-7, and monodansylcadaverine (MD) as indicated at pH 5.5 (A, 5), 7.4 (B), 7.4 (B). After 2.5 h at 0 °C, the specific binding of 125I-α-M5+ or 125I-TGF-β1 was determined. The specific binding of 125I-α-M5+ or 125I-TGF-β1 in the absence of trifluoperazine and other compounds was taken as 100% binding. Each data point is the average of quadruplicate determinations.

pH binding site-mediated or annexin VI-mediated binding in intracellular (endosomal) trafficking and degradation of internalized LRP ligands.
Acidic pH Binding Sites and Cell Surface Annexin VI

**Table III**

Effect of TFP on the degradation of cell surface-bound $^{125}$I-TGF-β, and $^{125}$I-α-M* in Mv1Lu cells

Cells were incubated with $^{125}$I-TGF-β, (100 ng) or $^{125}$I-α-M* (2 nm) at 10 μM TFP in the presence and absence of 200-fold excess of unlabelled TGF-β, or α-M* at 0°C for 2.5 h in DMEM, pH 7.4. The cells were then washed and warmed to 37°C. After 1 h at 37°C, cell surface-bound, internalized, and degraded fractions of $^{125}$I-α-M* were determined. The experiments were performed in quadruplicate. Data are represented as mean ± S.D.

| Cell type | TFP | -TFP | +TFP | -TFP | +TFP |
|-----------|-----|------|------|------|------|
| $^{125}$I-TGF-β | 2,428 ± 14 (45) | 2,410 ± 80 (46) | 761 ± 96 (49) | 750 ± 20 (48) |
| Internalized | 2,641 ± 40 (50) | 2,880 ± 41 (54) | 488 ± 79 (31) | 810 ± 35 (52) |
| Degraded | 256 ± 16 (5) | 0 | 307 ± 69 (20) | 0 |

**DISCUSSION**

Acidic pH binding sites have been demonstrated in many cell types using various ligands, including IGFBP-3, vascular endothelial cell growth factor, transferrin, ApoE, and many others (33–35, 56, 57). However, they have not been well characterized. Here we demonstrate that LRP-1 ligands bind at acidic pH in MEF, PEA-13, and Mv1Lu cells. Unlike LRP-1, the acidic pH binding sites are sensitive to heparin and chondroitin sulfate. We also provide evidence to suggest that cell surface annexin VI is involved in the acidic pH binding of LRP-1 ligands (e.g. IGFBP-3, TGF-β, and α-M*) and other proteins. The evidence includes 1) Annexin VI is a major protein identified in Triton X-100 extracts of bovine liver plasma membranes, which binds to the α-M*-Sepharose affinity column at acidic pH (pH 5 and 6) in a Ca²⁺-dependent manner. 2) Cell surface annexin VI is known to bind Ca²⁺+, heparin and chondroitin sulfate (36–39). The acidic pH binding of $^{125}$I-TGF-β or $^{125}$I-α-M* in cells is sensitive to calcium depletion, heparin and chondroitin sulfate. 3) The acidic pH binding of $^{125}$I-TGF-β in Mv1Lu cells or $^{125}$I-α-M* in MEF, PEA-13, and Mv1Lu cells can be prevented by preincubation of cells with anti-annexin VI IgG but not control IgG. 4) The acidic pH binding of $^{125}$I-α-M* in cells is effectively blocked by lactoferrin as well as non-LRP-1 ligands such as transferrin, but not γ-globulin and *Pseudomonas* exotoxin (an LRP-1 ligand), and 5) A431 cells (a human endometrial carcinoma cell line), which do not express annexin VI (58), do not exhibit anti-annexin VI IgG-sensitive acidic pH binding of $^{125}$I-α-M*.

Annexin VI is a member of a family of structurally homologous Ca²⁺-dependent phospholipid-binding proteins (37, 58). It is abundant in rat liver endosomes (49, 50, 52), localized in the apical endosomes in rat hepatocytes, and colocalizes with Igp120, a prelysosomal marker in normal rat kidney cells (37, 49–52). It has been implicated in the budding of clathrin-coated pits from plasma membranes (37, 59) and is involved in the trafficking of low density lipoprotein from endosomes to the prelysosomal compartment (48). It has also been shown to be able to form Ca²⁺ channels and insert into membranes at acidic pH or in the presence of 4 mM GTP (38, 60). Although annexin VI (like other types of annexins) lacks a signal sequence for secretion, it has been identified extracellularly where it can act as a receptor for chondroitin sulfate (37). The cell surface location of annexin VI may be due to its ability to insert into phospholipid bilayers (38, 59). Here, using immunofluorescent staining, we demonstrate that annexin VI is localized at the cell surface of MEF, PEA-13 and Mv1Lu cells and other cell types. We also show that pretreatment of cells with anti-annexin VI IgG partially or completely prevents $^{125}$I-TGF-β binding to cells at pH 6.4 or $^{125}$I-α-M* binding to cells at pH 6.4 and pH 7.4. Since LRP-1 is known to be responsible for α-M* binding at pH 7.4, these results suggest that cell surface annexin VI may function as a receptor (at acidic pH) and a co-receptor (at pH 7.4) for LRP-1 ligands (e.g. α-M*). This suggestion is supported by several observations: 1) The complete inhibition of $^{125}$I-α-M* binding to the acidic pH binding sites by treatment of cells with anti-annexin VI IgG in Mv1Lu, MEF and PEA-13 cells indicates that cell surface annexin VI mediates the acidic pH binding of $^{125}$I-α-M* in these cells. 2) Cell surface annexin VI is involved in ligand binding, internalization and degradation at acidic pH. 3) The partial inhibition of $^{125}$I-α-M* binding (at pH 7.4) to LRP-1 by treatment of cells with anti-annexin VI IgG suggests that cell surface annexin VI may function as a co-receptor for only a fraction of LRP-1 on the cell surface. This suggestion is supported by the observation that 40% of LRP-1 binding $^{125}$I-α-M* was immunoprecipitated by anti-annexin VI IgG. 4) Cellular heparan sulfate and chondroitin sulfate have been shown to be receptors for certain LRP-1 ligands (61–64). Removal of heparan sulfate or chondroitin sulfate from cells by enzymatic digestion appears to diminish the ability of the cells to internalize and degrade these LRP-1 ligands. Since annexin VI has been shown to bind heparin and chondroitin sulfate at the cell surface, we hypothesize that the heparan sulfate or chondroitin sulfate complex of cell surface annexin VI may serve as a co-receptor for these LRP-1 ligands. 5) The corresponding expression of both LRP-1 and annexin VI, as determined by Western blot analysis occurs in all cell types examined. For example, fibroblasts (MEF and NIH 3T3 cells) exhibit 3–5-fold higher amounts of both LRP-1 and annexin VI than epithelial cells (mink lung epithelial cells). Carcinoma cells (e.g. HCT116 cells) that lack or express very low levels of LRP-1 also produce no or very little annexin VI (58). Both annexin VI and LRP-1/Tgf-V are hypothesized to be candidates for tumor suppressor gene products (3–5, 7, 8, 58). 6) Cell surface annexin VI forms ternary complexes with $^{125}$I-α-M* and LRP-1, as shown by co-immunoprecipitation (at pH 7.4) of annexin VI and $^{125}$I-α-M* in MEF cells but not in PEA-13 cells. 7) α-M* has been shown to regulate N-methyl-D-aspartate receptor-mediated calcium influx in primary culture neurons (16). Since annexin VI and other annexin family mem-

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2 J. S. Huang, I.-H. Liu, and S. S. Huang, unpublished results.
Characterization of the Interactions of Insulin-like Growth Factor Binding Protein-3 with the Type V Transforming Growth Factor-

The acidic microenvironment within solid tumors may contribute to changes in cellular physiology and responses of tumor cells. We hypothesize that the acidic pH binding sites or cell surface annexin VI in tumor cells may partially substitute for certain receptors (e.g. LRP-1, transferrin receptor, and LDL receptor, which have optimal activity at neutral pH) under such acidic conditions. Although the acidic pH binding sites (cell surface annexin VI) are less efficient than LRP-1 (at pH 7.4) in mediating ligand internalization and degradation, their high density in cells may enable them to function (at acidic pH) as a significant receptor class, comparable to LRP-1 or other receptors at pH 7.4 (Table II). If this hypothesis is correct, annexin VI should be important in animal pathophysiology. However, annexin VI-null mutant mice have been shown to exhibit normal phenotypes (38, 59), suggesting that other annexin family members or other proteins may also be involved in the acidic pH ligand binding activity of cells. This possibility is supported by the observations: 1) A431 cells, which lack annexin VI, exhibit anti-annexin VI IgG-insensitive acidic pH ligand (αM*) binding and internalization activity. 2) Other annexins (annexin I, III, IV, and V) are also capable of directly interacting with 125I-αM* with an optimal pH of 5 as demonstrated by dot blot analysis, and 3) fibronectin has recently been shown to mediate the acidic pH (pH 5.5) binding of VEGF in cells (57, 70).

Trifluoperazine and fluphenazine, which are weak bases and have calmodulin antagonist activity, have been used as antipsychotic drugs. Their antipsychotic actions are believed to be mediated by their activity as dopamine receptor antagonists (71). Trifluoperazine has been shown to reversibly deplete 50% of cell surface αM* receptors at 30 μM (43). Thioridazine, a phenothiazine derivative, has been reported to inhibit cellular degradation of 125I-labeled epidermal growth factor (72). The mechanisms by which phenothiazine derivatives affect these cellular processes are unknown. The possible involvement of the weak basicity (raising the pH of extracellular vesicles or lysosomes) and calmodulin antagonist activity of these compounds at the concentrations generally used have been ruled out (43, 72). Here we demonstrate that trifluoperazine and fluphenazine are effective inhibitors of 125I-αM* binding to the acidic pH binding sites (e.g. annexin VI) and to LRP-1 (at pH 7.4) with IC50 values of 65–75 and 25–30 μM, respectively. Since weak base compounds (e.g. acidic orange) are capable of entering cells and accumulating in the intracellular acidic compartments such as endosomes at a few hundred-fold higher concentration than that in medium (55), the acidic pH ligand binding inhibitory activity of trifluoperazine and fluphenazine may be pharmacologically significant. In our studies, treatment of cells with 10 μM trifluoperazine or fluphenazine completely inhibits cellular degradation of cell surface-bound 125I-TGF-β3 or 125I-αM* following internalization. At 10 μM in the medium, trifluoperazine or fluphenazine, both weak bases, should be able to accumulate in the lumen of endosomes at concentrations that are effective in inhibiting 125I-TGF-β3 or 125I-αM*-annexin VI (or acidic pH binding site complex formation) and endosomal subsequent lysosomal targeting. Promethazine, another weakly basic phenothiazine, appears to be ineffective in blocking cellular degradation of 125I-TGF-β3 or 125I-αM* under the same experimental conditions, suggesting that the inhibition of the 125I-TGF-β3 or 125I-αM* degradation by trifluoperazine or fluphenazine is specific and is likely due to its newly identified annexin VI or acidic pH ligand binding inhibitory activity.

Endosomal signaling has recently been shown to play a pivotal role in several ligand receptor-mediated signaling cascade systems (73, 74). Inhibition of lysosomal targeting for degradation of ligands should logically enhance or prolong endosomal signaling mediated by the ligand-receptor complex. Trifluoperazine or related compounds may be useful agents for enhancing pharmacological actions of ligands, which are sensitive to these compounds and utilize endosomal signaling.

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