Purification and Characterization of the Human KDEL Receptor†

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ABSTRACT: Retention of soluble endoplasmic reticulum (ER) proteins is ensured by their continuous retrieval from subsequent compartments in the secretory pathway. Soluble ER proteins which escape to the Golgi apparatus bind to the KDEL receptor, a seven-transmembrane receptor, and are then returned to the endoplasmic reticulum. We have overexpressed the human KDEL receptor in insect cells using the baculovirus system. Infected cells accumulate large amounts of functional receptor as judged by a ligand binding assay. A hexahistidine-tagged version of the receptor could be purified in a single step to near homogeneity with high yield. After reconstitution of purified KDEL receptor into liposomes, a similar affinity and pH dependence for the binding of KDEL peptides was observed compared to the receptor in its natural environment, indicating that purified KDEL receptor is sufficient for specific and pH-sensitive binding of KDEL ligands. Determination of the receptor affinity in different lipid environments revealed that the receptor affinity is only slightly influenced by its lipid environment, suggesting that regulation of the receptor affinity by its surrounding lipids does not play a crucial role for the sorting of KDEL proteins.

Maintenance of the complex architecture of the secretory pathway depends on the correct localization of newly synthesized proteins. Moreover, proteins localized in the organelles of the secretory pathway have to be retained at their proper position, despite the continuous flow of proteins and lipids from the endoplasmic reticulum (ER)[1] to the cell surface. Resident proteins of the ER, which are essential for the folding, assembly, and maturation of secretory and membrane proteins, have a conserved carboxyl-terminal sequence, typically KDEL or HDEL (single-letter amino acid code), which is both necessary and sufficient for their retention in the ER (Munro & Pelham, 1987). Proteins bearing this signal are able to acquire Golgi-specific carbohydrate modifications, indicating that they are retained in the ER by continuous retrieval from a post-ER compartment (Dean & Pelham, 1990). Such proteins are believed to bind to a membrane receptor in the ER−Golgi intermediate compartment or in the Golgi apparatus and then enter a retrograde pathway which returns them to the ER. There, the receptor−ligand complexes must dissociate, resulting in the release of the KDEL proteins into the lumen of the ER. The empty KDEL receptor molecules then return to the Golgi apparatus to await further rounds of retrieval [reviewed by Pelham (1990)].

The HDEL receptor of *Saccharomyces cerevisiae* was identified by genetic means and is the product of the *ERD2* gene (Semenza et al., 1990). Additional receptor homologues have subsequently been identified from a wide variety of species, including humans. The amino acid sequences reveal a highly conserved pattern of seven predominantly hydrophobic segments which are likely to span the membrane (Figure 1).

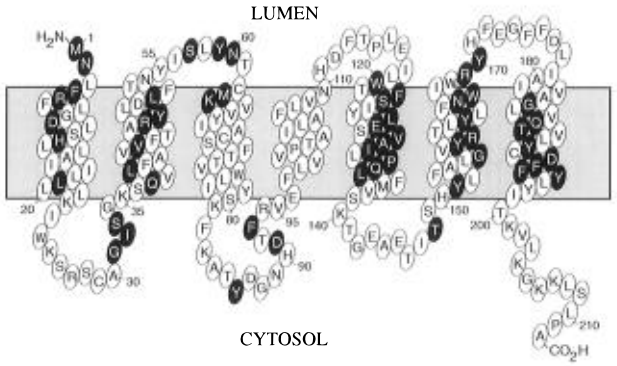

FIGURE 1: Predicted topology of the human KDEL receptor (Townsley et al., 1993). The sequence of human Erd2.1 is shown; residues in black are identical between the Erd2 genes of *H. sapiens* 1 (Lewis & Pelham, 1990), *H. sapiens* 2 (Lewis & Pelham, 1992b; Hsu et al., 1992), *S. cerevisiae* (Semenza et al., 1990), *K. lactis* (Lewis et al., 1990), *A. thaliana* (Lee et al., 1993), *P. falciparum* (Elmendorf & Haldar, 1993), *C. elegans* 1 (Accession Number Q09473 and D. Banfield, personal communication), *C. elegans* 2 (Accession Number P48583), and *D. melanogaster* (D. Banfield, personal communication). An alternative topology of the KDEL receptor has recently been proposed by Singh et al. (1993).

A direct interaction between KDEL peptides and the receptor has recently been shown using an *in vitro* assay (Wilson et al., 1993). These experiments demonstrated that binding *in vitro* exhibits the same sequence specificity as retention of soluble ER proteins *in vivo*. Furthermore, Wilson et al. (1993) showed that the binding affinity *in vitro* is strongly pH-dependent, suggesting a mechanism in which the binding and release of KDEL proteins may be controlled by the pH difference between the Golgi apparatus and the ER. As it is questionable whether the pH-difference between the ER and early Golgi compartments is large enough to account for the difference in ligand affinity required for efficient sorting, it seems likely that additional mechanisms

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[1] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; cpm, counts per minute; EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; K_D, dissociation constant; Ni-NTA, nickel nitrilotriacetic acid; PBS, phosphate-buffered saline, pH 7.4; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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are used to regulate the receptor−ligand interactions. In addition, it has remained unclear whether the KDEL receptor on its own is responsible for the binding characteristics displayed by Golgi membranes, although it has been shown that the KDEL receptor determines the capacity and specificity of the KDEL retrieval system (Semenza et al., 1990; Lewis et al., 1990; Lewis & Pelham, 1992a). To address these issues, we decided to purify the human KDEL receptor hErd2.1 (Lewis & Pelham, 1992b). This paper describes the expression, purification, and characterization of the human KDEL receptor using the baculovirus system.

MATERIALS AND METHODS

Materials. Chemicals, CHAPS, and insect cell medium (TNM-FH) were from Sigma. The baculovirus transfer vectors and the linearized baculovirus DNA (Baculogold) were purchased from Pharmingen. Phosphatidylcholine (egg yolk), phosphatidylinositol (bovine liver), and *E. coli* phospholipids (polar lipid extract) were from Avanti Polar Lipids Inc., Ni-NTA resin was from Qiagen.

Plasmids and Recombinant Viruses. The virus v-Erd2-myc was constructed and provided by Dr. M. Lewis. The plasmid pVL1393 without any insert was used to construct the control recombinant virus v-pVL (kindly donated by Dr. C. G. Tate). hErd2.1-wt was cloned from a pBluescript vector (from Dr. F. Townsley) into the *Sma*I/*Xba*I-digested baculovirus transfer vector pVL1393. To introduce a hexahistidine tag at the carboxyl terminus of the KDEL receptor after the myc-epitope, a plasmid containing the hErd2.1 cDNA with two restriction sites (*Eco*RI and *Xba*I) 3′ of the myc-encoding region was used (provided by Dr. M. Lewis). Synthetic oligonucleotides encoding the carboxyl-terminal hexahistidine tag and a stop codon were annealed and ligated into these sites. The oligonucleotides used had the following sequence (5′-3′): AATTCCCACCACCACCACCACCACTGAT and CTAGATCAGTGGTGGTGGTGGTGGTGGG (the partial restriction sites are underlined). The cDNA encoding the histidine-tagged receptor (hErd2.1-myc-his) was then cloned into the *Sma*I/*Bgl*II-digested baculovirus transfer vector pVL1393 (see Table 1 for amino acid sequences). All sequences were confirmed by dideoxy sequencing.

Preparation of Virus and Expression of Recombinant Proteins in Sf9 Cells. Construction of recombinant baculoviruses and maintenance of Sf9 insect cell cultures were performed as described (Summers & Smith, 1987; Tate & Blakely, 1994). Cells were grown at 27 °C in TNM-FH medium supplemented with 10% fetal calf serum. Expression studies were performed in 75-cm^2 flasks (adherent) for ligand binding experiments or in spinner flasks (50−500 mL of cell suspension) for receptor purification. The virus titer was determined by end point dilution, and infections were performed at a multiplicity of infection of 10.

Membrane Preparations and Ligand Binding Assays. Unless otherwise mentioned, infected cells were harvested 48 h postinfection by centrifugation at 1500*g* for 2 min. The cell pellet was washed 3 times with cold PBS and lysed by sonication (Soniprep 150, Sanyo, UK, 3 × 3 s pulses) in 20 mM Hepes, pH 7.4, 1 M NaCl, 2 mM EDTA, and 1 mM β-mercaptoethanol. The homogenate was spun at 500*g* for 10 min to remove unbroken cells, and a crude membrane fraction was recovered from the supernatant by spinning at 100000*g* for 20 min at 4 °C. The membranes were resuspended in 10 mM Hepes, pH 7.4, for ligand binding assays or in PBS containing 200 mM sucrose for protein purification and stored frozen at −70 °C without loss of activity. Carbonate-stripped Golgi membranes from rat liver were prepared as described (Tabas & Kornfeld, 1979; Wilson et al., 1993).

In vitro binding assays with membranes (0.5−1.0 μg of protein) were performed using 2 × 10^5 cpm of ^{125}I-labeled YTSEKDEL peptide in the presence of excess unlabeled peptide (YTSEKDELGL or YTSEKDEL) in cacodylate buffer, pH 5.0, as described (Wilson et al., 1993). For Scatchard analysis, membranes (approximately 200 ng of protein) were permeabilized with 0.3% CHAPS in 10 mM Hepes, pH 7.4, prior to incubation with labeled YTSEKDEL peptide with a specific activity of 15 000 cpm/ng. Unless otherwise mentioned, specific binding was determined by the difference between a binding reaction which contained 100 ng of unlabeled YTSEKDELGL peptide (nonfunctional ligand, total binding) and one which contained 100 ng of unlabeled YTSEKDEL peptide (functional ligand, background binding).

Receptor Purification and Reconstitution. All experiments were carried out at 4 °C. Membrane preparations (15−20 mg of protein) containing hexahistidine-tagged KDEL receptor were solubilized at a protein concentration of 2 mg/mL in solubilization buffer [50 mM sodium phosphate, pH 7.5, 1 M NaCl, 20% glycerol, 1 mM β-mercaptoethanol, and 2% CHAPS (w/v) containing 3 μg/mL leupeptin, 2 mM benzamidine, 1 mM PMSF, and 1 mM EDTA] overnight with rotation. The sample was then clarified by spinning for 1 h at 100000*g*, and the supernatant was subsequently incubated for 3 h with 1 mL of Ni-NTA resin, preequilibrated in wash buffer (identical to solubilization buffer, but contained 0.5% CHAPS and 0.2 mg/mL phospholipids). Unbound protein was removed by washing with 5 mL of wash buffer, followed by a second washing step with identical buffer containing 35 mM imidazole. Bound protein was eluted with wash buffer containing 200 mM imidazole. The eluted fractions were analyzed by SDS−PAGE.

Reconstitutions were carried out by detergent removal using dialysis. The pooled fractions, containing purified KDEL receptor, were mixed with bath-sonicated phospholipids, solubilized in 10 mM Hepes, pH 7.4, containing 5 mM DTT and 2% CHAPS, at a final concentration of 3.4 mg/mL phospholipid. The sample was then dialyzed for 12−24 h against PBS with one change of buffer. The proteoliposomes were collected by centrifugation (20 min at 100000*g*), resuspended in 10 mM Hepes, pH 7.4, and stored at −70 °C.

Miscellaneous Procedures. Discontinuous SDS−PAGE was performed as described elsewhere (Laemmli, 1970). Protein samples were incubated in SDS-loading buffer at 37 °C for 15 min prior to loading on a gel. Protein staining with silver nitrate following SDS−PAGE was performed as described by Wray et al. (1974). Immunoblotting was performed using the ECL chemiluminescence detection system (Amersham International, Amersham, U.K.). Protein concentrations were determined by the method of Bradford (1976) or by staining with Amido Black (Schaffner & Weissmann, 1973). Quantitation of autoradiographs was performed using a Model 300A densitometer (Molecular Dynamics, Sunnyvale, CA).

Table 1: Carboxyl-Terminal Amino Acid Sequences of KDEL Receptor Constructs Used in This Study[a]

plasmid	baculovirus construct	carboxyl-terminal sequence
(A) hErd2.1-wt	v-Erd2-wt	KKLSLPA
(B) hErd2.1-myc	v-Erd2-myc	KKLSLPAPCMEQKLISEEDLN
(C) hErd2.1-myc-his	v-Erd2-myc-his	KKLSLPATMEQKLISEEDLNSHHHHHH
(D) −	v-pVL	no insert

[a] (A) Untagged human Erd2.1 (hErd2.1-wt); (B) human Erd2.1 with a c-myc epitope at the carboxyl terminus (hErd2.1-myc); (C) human Erd2.1 with six histidine residues C-terminal to the c-myc epitope (hErd2.1-myc-his); (D) recombinant control virus (v-pVL). The nomenclature for the corresponding recombinant baculoviruses and the amino acid sequences (single-letter amino acid code) are shown on the right. The c-myc epitope is underlined.

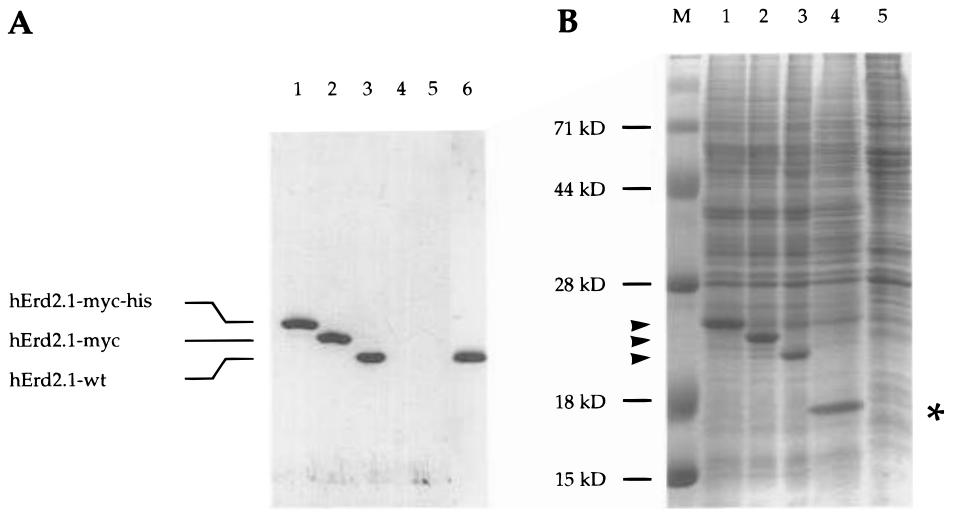

FIGURE 2: Analysis of KDEL receptor expression in Sf9 cells. Membranes prepared from cells infected with different recombinant baculoviruses and Golgi membranes prepared from rat liver were analyzed by immunoblot (A) and SDS−PAGE (B, Coomassie blue staining). The immunoblot was probed with an anti-Erd2 antibody. The lanes in panels A and B contain membranes from infections with (1) v-Erd2-myc-his, (2) v-Erd2-myc, (3) v-Erd2-wt, (4) v-pVL, (5) noninfected cells, and (6) Golgi membranes prepared from rat liver. The positions of the expressed receptor proteins are labeled. The protein in panel B, lane 4 (*asterisk*), is a truncated version of polyhedrin, expressed by the control virus. The positions of molecular size markers (M) are shown.

RESULTS

We used the baculovirus system to express the human KDEL receptor Erd2.1 in insect cells, as this system has successfully been used to overexpress many eukaryotic membrane proteins (Grisshammer & Tate, 1995). The cDNAs encoding different tagged versions of the human KDEL receptor (Table 1) were introduced into the *A. californica* nuclear polyhydrosis virus genome under the control of the polyhedrin promoter. The receptor with a myc-tag at the carboxyl terminus has previously been shown to function *in vivo* (Semenza et al., 1990; Lewis & Pelham, 1992a). A hexahistidine-tag was introduced C-terminal to the myc-tag in order to facilitate affinity purification using nickel affinity chromatography.

Expression of the Human KDEL Receptor in Insect Cells. Western blots of membranes prepared from infected cells were probed with an anti-Erd2 antibody (Sönnichsen et al., 1994) and revealed a band of the predicted molecular mass of 25 kD (Figure 2A, lanes 1−3). The immunoreactive band in cells expressing untagged receptor (Figure 2A, lane 3) comigrates with the receptor from rat liver Golgi membranes (Figure 2A, lane 6). The antibody was monospecific for Erd2 as it does not react with other proteins in rat liver Golgi-enriched membranes, nor with membrane proteins prepared from either cells infected with the control virus (Figure 2A, lane 4) or uninfected cells (lane 5). The addition of a myc- (Erd2-myc, lane 2) or a myc- and a his-tag (Erd2-myc-his, lane 1) causes a characteristic reduction of the electrophoretic mobility. Erd2 is easily identified on a Coomassie-stained SDS-gel (Figure 2B), where the same pattern of bands can be observed. The KDEL receptor is one of the most abundant proteins in these crude membrane fractions.

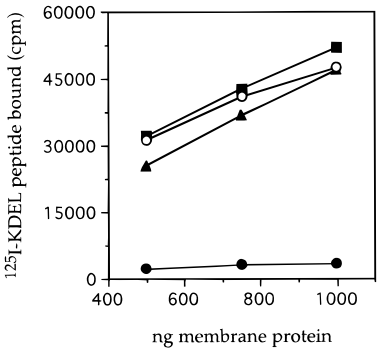

FIGURE 3: KDEL receptor expressed in Sf9 cells binds KDEL peptides *in vitro*. Cells were infected with v-Erd2-myc-his (▲), v-Erd2-myc (■), v-Erd2-wt (○), or the control virus v-pVL (●) and harvested after 48 h, and the prepared membrane fractions were assayed for specific binding of ^{125}I-YTSEKDEL. The specific binding activity was 85−90% of the total binding activity. Results are the mean of 2 experiments performed in duplicate.

KDEL Binding Activity. The activity of the expressed Erd2 was examined using a recently developed *in vitro* ligand binding assay (Wilson et al., 1993). Membranes prepared from infected cells show specific binding for KDEL-containing peptides (Erd2-wt, Erd2-myc, or Erd2-myc-his), whereas control membranes (pVL) show no detectable binding activity (Figure 3). A time course of the expression of myc-tagged Erd2 is shown in Figure 4. The appearance

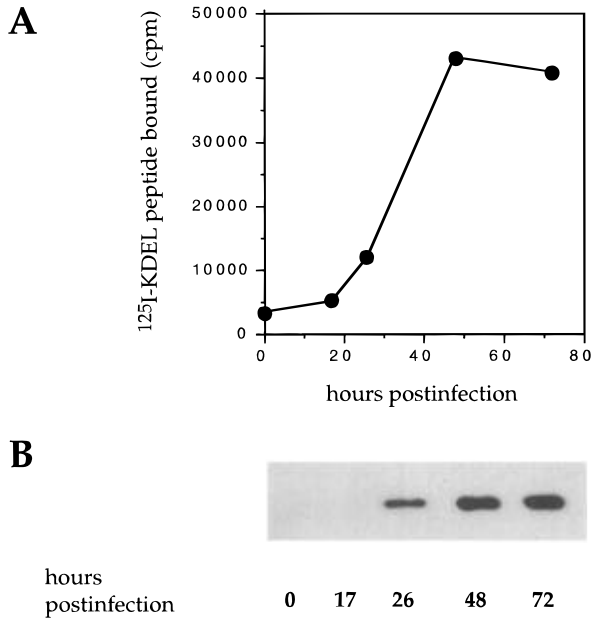

FIGURE 4: Time course of expression of the receptor protein and of KDEL binding activity in insect cells. Membranes were prepared from v-Erd2-myc-infected cells at different time points postinfection and assayed by ligand binding assay (A) or Western blot (B). The blot was probed with a monoclonal anti-myc antibody (9E10).

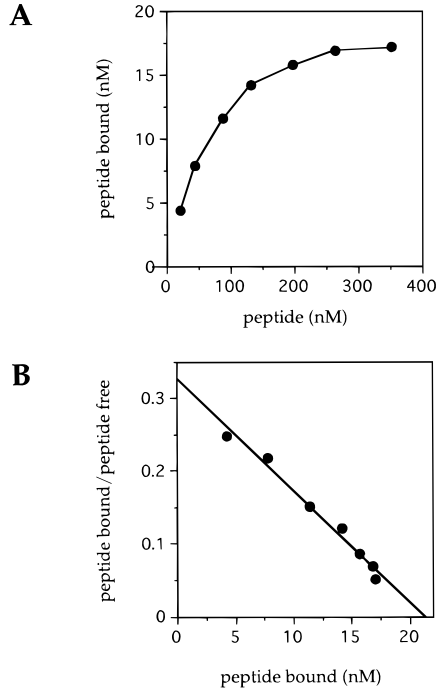

FIGURE 5: Ligand binding characteristics of the KDEL receptor expressed in Sf9 cells. (A) Binding curve of ^{125}I-YTSEKDEL to membranes prepared 48 h postinfection from cells infected with v-Erd2-myc. Aliquots of membranes, preincubated in 0.3% CHAPS to permeabilize the membranes, were incubated with increasing amounts of ^{125}I-YTSEKDEL, and the specific binding was determined. The results shown are from a typical experiment performed in duplicate. (B) Scatchard analysis of the data shown in panel A. The data are given in Table 2.

of ligand binding activity correlated with the expression of Erd2 with maximal activity 48 h postinfection. As the binding activity of endogenous receptor of the Sf9 cells is insignificant (see Figure 3, control membranes), these experiments demonstrate that the human KDEL receptor expressed in Sf9 cells is functional and that the measured binding activity is entirely due to the recombinant Erd2. To determine the binding affinity and the number of total binding sites, a Scatchard analysis was performed (Figure 5). These analyses revealed a K_D of 80 nM for the myc-tagged KDEL receptor. Moreover, the fusions to the carboxyl terminus do not affect the binding affinity of the receptor *in vitro* significantly (Table 2). Thus, the human KDEL receptor expressed in Sf9 cells shows the same affinity for KDEL peptides as the receptor from rat liver Golgi (Table 3). These experiments also show that active receptor molecules constitute approximately 7% (by weight, Table 2) of the membrane proteins in high-salt-stripped membrane fractions, which corresponds to approximately 75 million binding sites per cell or 7 mg of functional receptor per liter of cell culture. To investigate the specific activity of the expressed Erd2, we used anti-Erd2 antiserum to compare the Erd2 content of crude membranes from Sf9 cells expressing hErd2-wt and rat liver Golgi membranes, assuming that the receptor in rat liver Golgi membranes is 100% active. *In vitro* binding experiments using these membranes revealed that approximately 30−50% of the expressed receptor protein is functional.

Receptor Purification. The KDEL receptor could be solubilized using various detergents. As the binding activity of the receptor could not be reliably measured in solution, the receptor was reconstituted into phosphatidylcholine liposomes to investigate the detergent optimal for preserving the receptor activity. Solubilization in 2% CHAPS was proven to be best for preventing loss of binding activity. Approximately 40% of the total Erd2 could be solubilized under these conditions. The histidine-tagged receptor solubilized from crude membrane preparations was purified using nickel affinity chromatography (Figure 6). The column flow-through indicated that around 90% of the receptor was bound to the Ni^{2+}-containing resin (Figure 6B), whereas the bulk of proteins flowed through the column (Figure 6A, lane 4). Weakly bound proteins were removed by washing steps containing 35 mM imidazole, and Erd2 was eluted with 200 mM imidazole. The eluted fraction was reconstituted into phosphatidylcholine liposomes by detergent removal (Figure 6A, lanes 5 and 6). The reconstituted KDEL receptor was greater than 90% pure based on Coomassie staining (Figure 6A, lane 6). The identity of the KDEL receptor was verified by the amino-terminal sequence (data not shown) and Western blotting (Figure 6B). In a typical experiment, the overall yield of purified protein was 200 μg from 20 mg of Sf9 membrane protein, which is obtained from a 200 mL suspension culture.

Table 2: Determination of K_D and Expression Levels for Human KDEL Receptors Expressed in Insect Cells

recombinant baculovirus[a]	dissociation constant[b] (nM)	expression level[bc]
v-Erd2-wt	85 ± 36	6.2 ± 2.1
v-Erd2-myc	80 ± 29	7.5 ± 2.5
v-Erd2-myc-his	77 ± 37	6.1 ± 1.6

[a] See Table 1 for nomenclature. [b] Figures represent the average of 5−10 independent experiments performed in duplicate; errors are given as SE. [c] Micrograms of Erd2 per microgram of total membrane protein × 100.

Characterization of Purified Receptor Preparation. To investigate the functionality of the purified receptor, *in vitro* binding analyses using purified receptor were performed. In

Table 3: KDEL Binding Characteristics of the Rat KDEL Receptor from Rat Liver Golgi Membranes, the Human KDEL Receptor (hErd2-myc-his) Expressed in Insect Cells, and Purified Human KDEL Receptor (hErd2-myc-his) in Liposomes

receptor material	dissociation constant[a] (nM)	pH of maximal KDEL binding[b]
Golgi membranes[c]	75	5
crude Sf9 membranes[d]	77 ± 37	5
reconstituted receptor (phosphatidylcholine)[e]	45 ± 8	5
reconstituted receptor (phosphatidylcholine/phosphatidylinositol)[f]	75 ± 14	nd[h]
reconstituted receptor (*E. coli* phospholipids)[g]	167 ± 39	nd[h]

[a] Values represent the average of 3−8 experiments performed in duplicate; errors are given as SE. [b] KDEL binding was tested from pH 4.5 to pH 7.0. [c] Rat liver Golgi membranes [data taken from Wilson et al. (1993)]. [d] Crude membranes prepared from cells expressing hErd2-myc-his. [e] Purified Erd2-myc-his, reconstituted into phosphatidylcholine liposomes. [f] Purified Erd2-myc-his, reconstituted into phosphatidylcholine/phosphatidylinositol liposomes (molar ratio of 9:1). [g] Purified Erd2-myc-his, reconstituted into *E. coli* phospholipid liposomes. [h] Not determined.

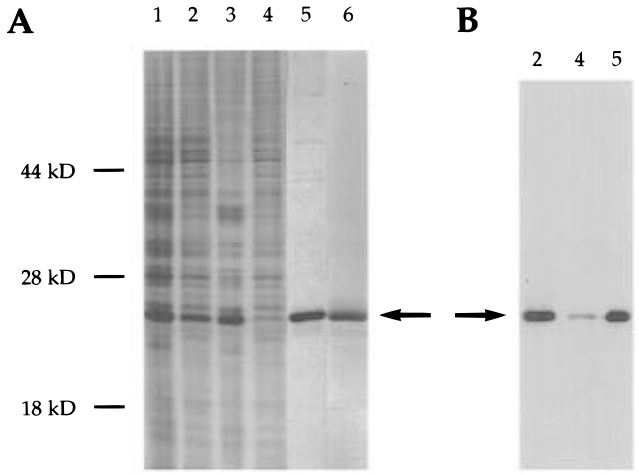

FIGURE 6: Purification of hErd2-myc-his on Ni-NTA. Membranes from infected cells (v-Erd2-myc-his) were extracted with CHAPS, and the soluble fraction was loaded onto a Ni-NTA column and chromatographed as described under Materials and Methods. Fractions were analyzed by SDS−PAGE (A, silver staining, except lane 6, Coomassie blue staining) and immunoblot (B, probed with anti-myc antibody 9E10). Lane 1, membranes; lane 2, soluble CHAPS extract ("load"); lane 3, insoluble CHAPS extract; lane 4, flow-through; lanes 5 and 6, eluted fractions, reconstituted into phosphatidylcholine liposomes. The positions of the receptor protein and of molecular mass standards are indicated.

a typical binding experiment (see Materials and Methods), phosphatidylcholine liposomes containing purified KDEL receptor bind approximately 50 000 cpm of ^{125}I-YTSEKDEL specifically, whereas pure phosphatidylcholine liposomes containing comparable amounts of phosphatidylcholine but lacking Erd2 bind only insignificant amounts of ^{125}I-YTSEKDEL (approximately 1000 cpm). Moreover, the purified material shows the same binding characteristics as the crude starting material from infected Sf9 cells (Table 3, Figure 7) and as rat liver Golgi membranes (Table 3): both the binding affinity and the effect of pH on binding are very similar. Binding assays performed in the absence and presence of low concentrations of detergent suggest that the receptor is found in both orientations in the proteoliposomes. To investigate whether any loss of binding activity occurred during the solubilization and purification procedures, we compared the binding activity of crude Sf9 cell membranes to purified receptor in liposomes containing identical amounts of hErd2-myc-his as judged by quantitative Western blotting. These experiments showed that no significant losses of binding activity occurred as typically 95% of the specific binding activity (binding per arbitrary unit of Erd2) could be recovered.

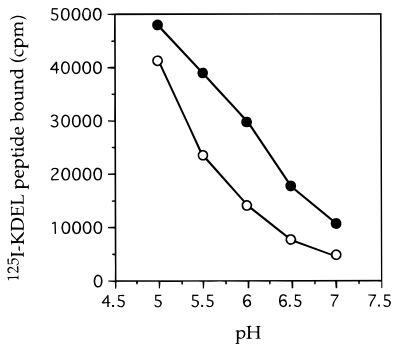

FIGURE 7: pH sensitivity of KDEL binding. The binding activity of the KDEL receptor (hErd2-myc-his) in a crude membrane fraction derived from infected Sf9 cells (○) and of purified receptor in phosphatidylcholine liposomes (●) was determined at different pHs; 50 mM sodium cacodylate was adjusted to the appropriate pH; each point represents the mean of 3 experiments performed in duplicate. The data are shown without background subtraction.

Activity of the Receptor in Different Lipids. Efficient release of bound KDEL proteins into the lumen of the endoplasmic reticulum is essential for a proper functioning of the KDEL retrieval system. Wilson et al. (1993) have shown that the binding affinity of the KDEL receptor is pH-dependent, with a maximum at acidic pH. Thus, a neutral pH in the lumen of the ER would facilitate the release of KDEL proteins. As additional factors are likely to contribute to the regulation of the receptor affinity, we investigated whether the lipid environment of the receptor affects its affinity for KDEL ligands. Lipids are believed to play an important role in protein sorting (Bretscher & Munro, 1993; Liscovitch & Cantley, 1995); we therefore measured the affinity of purified receptor in different lipid environments. Phosphatidylcholine, a neutral lipid, has been shown to be the major lipid of the ER, whereas phosphatidylinositol is found in smaller quantities in the ER (van Meer, 1989). The polar lipid extract from *E. coli* contains both neutral and acidic phospholipids (70% phosphatidylethanolamine, 15% phosphatidylglycerol, 15% cardiolipin; Chen & Wilson, 1984). Table 3 shows that the affinity of the human KDEL receptor varies in different lipid environments. The K_D of the receptor in crude Sf9 cell membranes and of purified receptor in phosphatidylcholine/phosphatidylinositol liposomes (77 nM and 75 nM, respectively) is identical to the K_D of rat liver Golgi membranes (75 nM). In phosphatidylcholine liposomes, the affinity of purified receptor is slightly higher (45 nM) and approximately 2-fold lower in liposomes formed with *E. coli* phospholipids (167 nM).

DISCUSSION

The results in this paper describe the first functional purification and biochemical characterization of the human KDEL receptor. Insect cells infected with a recombinant baculovirus encoding the human KDEL receptor accumulated approximately 2.7 nmol of active receptor/mg of membrane protein. This is one of the highest levels of overexpression of a functional polytopic membrane protein ever achieved with the baculovirus system (Grisshammer & Tate, 1995). One of the possible explanations for the high levels of expression achieved in this study is that posttranslational modifications such as glycosylation are not required for activity. These experiments showed that the fusion of peptide tags to the carboxyl terminus of the receptor does not significantly alter the receptor affinity. However, the addition of tags to the carboxyl terminus does alter the kinetics of vesicular transport of the receptor *in vivo* (M. Lewis, personal communication): it has been shown that overexpression of lysozyme−KDEL results in a movement of the human myc-tagged receptor from the Golgi to the ER in COS cells (Lewis & Pelham, 1992a). Under these conditions, the endogenous monkey receptor can only be shifted to punctate structures, which are likely to represent intermediate structures in the anterograde pathway (M. Lewis, personal communication). Thus, additional amino acids at the C-terminus seem to alter the rate of receptor recycling (Townsley et al., 1994), either by accelerating retrograde or by slowing down anterograde transport, which leads to a shift in receptor localization at a steady-state level.

A rapid, single-step purification to near-homogeneity was developed, yielding approximately 200 μg of almost pure KDEL receptor from a 200 mL cell culture. The purified, reconstituted receptor was active and showed very similar *in vitro* binding properties to those of rat liver Golgi membranes: the dissociation constant was approximately 45 nM, and the binding was sensitive to the pH with maximal binding at pH 5. As no significant losses of receptor activity occurred during the solubilization, purification, and reconstitution procedures, the specific activity of purified material (binding activity per arbitrary unit of Erd2) was essentially identical to the activity of the starting material, which was estimated to be 40% active.[2] From our purification, we therefore conclude that the KDEL receptor alone can account for the KDEL binding activity of Golgi membranes and that the binding characteristics displayed are an intrinsic property of the receptor protein.

A prerequisite for the sorting of KDEL proteins is a mechanism which ensures efficient binding of ligand in the Golgi apparatus and efficient release of ligand in the ER. Previous studies have shown that ligand binding is pH-sensitive and optimal at acidic pH (Wilson et al., 1993), suggesting a model where the pH differences between ER and Golgi are facilitating the sorting of KDEL proteins. Although the pH of the Golgi apparatus is believed to be acidic compared to the pH of the ER lumen (Anderson & Pathak, 1985; Seksek et al., 1995), it is doubtful whether the difference between early Golgi compartments and the ER is enough to ensure efficient sorting. The release of the ligand in the ER is a particular problem, as the concentration of KDEL proteins in the ER is in the range of millimolar (Booth & Koch, 1989). As there is increasing evidence for an important role of lipids in vesicular transport (Brown & Rose, 1992; Cleeves et al., 1991; Liscovitch & Cantley, 1995), we investigated a possible function of lipids in modulating the affinity of the KDEL receptor. This was based on the proposal that the lipid compositions of the Golgi apparatus and the ER are believed to be different (van Meer, 1989), e.g., with a higher cholesterol concentration in the Golgi apparatus compared to the ER. The receptor protein may require the specific lipid environment of the Golgi apparatus to bind KDEL proteins with high affinity, whereas an unfavorable environment in the ER would decrease its affinity and therefore facilitate the release of the ligand. Several specific lipid compositions were tested, and the results demonstrate that the receptor does not require levels of cholesterol normally found in the Golgi apparatus of higher eukaryotic cells to bind KDEL ligands with high affinity: the affinity of the receptor in crude Sf9 cell membranes, which contain 10-fold lower levels of cholesterol than found in animal cells (Gimpl et al., 1995), in phosphatidylcholine liposomes, and in phosphatidylcholine/phosphatidylinositol liposomes is very similar to the affinity found in rat liver Golgi membranes. But even in *E. coli* phospholipids, which show a very different lipid composition compared to eukaryotic cells as they contain no significant amounts of cholesterol and phosphatidylcholine (Chen & Wilson, 1984), the receptor affinity of 167 nM is only 2-fold lower than the affinity of the (rat) receptor in Golgi membranes (75 nM). These experiments suggest that although the affinity of the KDEL receptor is influenced by the lipids, the receptor does not require a specific lipid composition to bind KDEL ligands with high affinity. Given the large difference in KDEL protein concentration between the ER and the Golgi apparatus, we believe that changes in K_D in the order of magnitude we observed in the different lipids tested are not sufficient to contribute significantly to the release of KDEL proteins in the ER.

What other factors could have effects upon binding? Different ionic conditions have already been shown not to alter the specific binding *in vitro* (Wilson et al., 1993). Limited solubility of KDEL proteins (Booth & Koch, 1989) or masking of the KDEL sequence *in vivo* may lower the apparent KDEL protein concentration, which is "seen" by the receptor in a significant way. So far, no detailed binding studies using KDEL proteins rather than short peptides have been performed. Alternatively, other proteins that interact with the KDEL receptor may modulate its ligand binding properties. For example, recent evidence suggests that the receptor leaves the ER in COPII-coated vesicles, and leaves the Golgi apparatus in COPI-coated vesicles (Lewis & Pelham, 1996). If binding by COPI and COPII coat proteins was sufficient to stabilize high-affinity and low-affinity receptor conformations, respectively, directed transport of ligand could be achieved. A preferred and rapid incorporation of unoccupied receptor into forward (COPII-coated) transport vesicles combined with a high off-rate of the ligand in the ER may ensure efficient release of KDEL ligand into the ER lumen. Strikingly, we have observed a high off-rate for KDEL peptides *in vitro* (the half-time of dissociation is approximately 15 s; A. A. Scheel, unpublished observation).

[2] The peptide used to raise the anti-Erd2 antiserum (Sönnichsen et al., 1994) is conserved between the two human receptor isoforms (Lewis & Pelham, 1992b) and the bovine receptor (Tang et al., 1993). As the sequence of the rat receptor is unknown, it is unclear whether the antibody recognizes the rat receptor with the same affinity.

In conclusion, the first characterization of purified, functional human KDEL is reported. The availability of purified receptor will allow us to analyze the possible effects of coat proteins and other proteins on modulating the ligand binding affinity of the KDEL receptor.

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