JOURNAL OF CELLULAR PHYSIOLOGY 142:574–580 (1990)

Internalization of the Fibronectin Receptor Is a Constitutive Process

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Using a monoclonal antibody specific for the hamster fibronectin receptor (FnR), we have demonstrated that a portion of the CHO cell FnR population is constitutively endocytosed. Three independent techniques were used to study the internalization: 1) after saturation binding of an anti-FnR antibody (PB1) to cells at 4°C, internalization was initiated by warming to 37°C, and then acid/salt elution of membrane-bound ligand was used to quantitate the internalized ^{125}I-PB1; 2) cell vesicular traffic was pharmacologically disrupted with monensin or chloroquine, and the subsequent reduction of the cell surface pool of FnR was monitored; and 3) selective immunoprecipitation was used to separate surface and internalized ^{125}I-labeled FnR. These experiments indicate that about 30% of the cell surface FnR is endocytosed with a $t_{1/2}$ of 7 min and that this internalization occurs regardless of the ligation state of the receptor. Other observations indicate that the larger fraction of the cell surface FnR pool (70–75%) is apparently shed from the cell upon ligation with antibody at 37°C. This process occurs much more slowly than receptor internalization and leads to an overall reduction in the amount of cell surface FnR. Our results suggest physically or chemically distinct populations of FnR, one of which is unavailable for internalization and recycling.

Endocytosis provides a mechanism for cellular uptake of nutrients and internalization of receptor-bound ligands such as hormones and growth factors (for reviews, see Steinman et al., 1983; Mellman et al., 1986; Stahl and Schwartz, 1986; Forgac, 1988). Many receptors recycle between the plasma membrane and the endosome compartment, thus enhancing the overall efficiency of the uptake process. Very often, receptor internalization and recycling occurs even in the absence of ligand (e.g., LDL, Fc, and transferrin receptors) as suggested by the depletion of receptors from the cell surface in the presence of compounds like monensin or chloroquine which are known to disrupt vesicular traffic (Mellman et al., 1986). This constitutive recycling is thought to be due to the continuous, non-specific pinocytic uptake of the plasma membrane. For macrophages, this results in the internalization of about 200% of the plasma membrane surface area/hour; for fibroblasts and *Dictyostelium discoideum,* the rate is between 50–100%/h (Steinman et al., 1976; Thilo and Vogel, 1980). These numbers extrapolate into a basal rate of receptor turnover of once every 0.5–2 h or faster, depending upon whether the particular receptors are present in coated pits even in the absence of ligand or whether ligation increases the rate of the receptor's movement into the coated regions (Mellman et al., 1986). Once internalized, most receptors seem to move back to the cell surface at about the same rate. In macrophages, the $t_{1/2}$ for the reappearance of transferrin, α-macroglobulin-trypsin, and mannose terminal glycoprotein receptors on the cell surface is about 2 min, regardless of whether ligated at the time of internalization (Ward et al., 1989). McKinley and Wiley (1988) have recently suggested that the constitutive internalization of coated pits is the major mechanism by which membrane is removed in fibroblastic cells.

The mammalian fibronectin receptor (FnR) is a member of the integrin superfamily of cell surface proteins, several of which have a binding specificity for proteins containing arg-gly-asp (RGD) in their cell-reactive domains. Like other integrins, the FnR comprises 2 non-covalently linked glycoprotein subunits termed α and β; the FnR belongs to the β_1 group of integrins, and the human receptor is designated $\alpha_5\beta_1$. For reviews of integrin structure, binding specificities, and distribution, see Juliano (1987), Buck and Horwitz (1987), Hynes (1987), Ruoslahti and Pierschbacher (1987), and Ruoslahti (1988).

Previously, other investigators have provided indirect evidence suggesting that the FnR is internalized. McAbee and Grinnell (1985) showed protease-treated BHK cells were able to recover their ability to bind and phagocytize Fn-coated latex beads without the benefit of protein synthesis. Later, Molnar and co-workers (1987) demonstrated that phagocytosis of Fn-coated latex beads was mediated by a molecule that is sensitive to pronase and is depleted from the cell surface by chloroquine. Additionally, Grinnell (1986) has reported endocytosis of substratum-bound Fn; Avnur and Geiger (1981) used rhodamine-labeled Fn to demonstrate a transient association of Fn with clathrin-containing structures.

Received August 7, 1989; accepted November 1, 1989.

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In this report, we present evidence for the endocytosis of a portion of the FnR population in CHO fibroblasts in a manner consistent with the constitutive internalization of the plasma membrane. Using the FnR-specific monoclonal antibody PB1 as a ligand and gauge of FnR density, we show by three independent techniques that about 30% of the total FnR population can be internalized regardless of the state of ligand occupation. We also note a larger fraction of receptor that seems to be lost from the surface by a much slower process.

MATERIALS AND METHODS

Materials

$Na^{125}I$ (specific activity of 70 mCi/mg) was purchased from ICN (Irvine, CA). Monensin, chloroquine, cycloheximide, tunicamycin, and molecular weight standards were from Sigma (St. Louis). Dibutyl phthalate was supplied by Aldrich, and bovine serum albumin (BSA, fraction V) was from Collaborative Research (Lexington, MA). HEPES buffer was purchased as a sterile 1 M stock from Gibco. Protein A coupled to Sepharose CL-4B was purchased from Pharmacia. Rabbit anti-mouse IgG for use as a secondary antibody was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cell culture and maintenance

Chinese hamster ovary (CHO) fibroblastic cells were grown and maintained in suspension culture in α-MEM (Gibco) with 5% fetal calf serum or 10% bovine calf serum supplemented with growth factors (Hyclone) as described earlier (Harper and Juliano, 1980).

Antibody production and affinity matrix preparation

The monoclonal antibodies PB1 and 7E2 were purified from mouse ascites and used as the purified IgGs as described by Brown and Juliano (1988). Monoclonal PB1 is specific for the intact hamster FnR (the hamster equivalent of human integrin $\alpha_5\beta_1$) and inhibits cell adhesion to Fn; monoclonal 7E2 recognizes the β_1 subunit. For some immunoprecipitation experiments, PB1 or 7E2 was coupled to Affi-gel 10 (Bio-Rad Laboratories, Richmond, CA) as suggested by the manufacturer, usually at a concentration of 10 mg antibody/ml Affi-gel 10.

Iodination of monoclonal PB1 and cell surface proteins

Iodogen (Pierce Chemical Co., Rockford, IL) was used to radiolabel the monoclonal PB1 (1 mCi ^{125}I/mg protein) for use in binding assays. CHO cell surface proteins were labeled using the same reagent at a ratio of 1 mCi $^{125}I/5 \times 10^7$ cells (Brown and Juliano, 1986). Cell viability after iodination was determined by trypan blue exclusion and cell counts and was found to be about 90%. TCA-precipitable and -soluble counts were assayed as described by Goding (1986).

Receptor binding assays

For binding assays, suspension-grown CHO cells were washed 3× with α-MEM and resuspended at a density of $2–4 \times 10^6$ cells/ml in binding medium (α-MEM with 1% BSA and 20 mM HEPES, pH 7.3). ELISA studies with anti-Fn antibody indicate that this extensive washing removes Fn bound to the cells during culture (C. Schreiner, unpublished results); thus, all cell surface PB1 binding sites are exposed. The ligand, ^{125}I-PB1, was diluted in binding medium and added to the cells. The reaction was performed at either 4°C or 37°C with continuous rocking on a Nutator (Adams). Non-specific binding (routinely <10%) was determined by performing an identical assay with 100-fold excess unlabeled PB1 added. Specific binding was then calculated by subtracting the non-specific binding from the total binding.

The binding reaction was terminated, and the free and bound ligand were separated by transferring 100 μl aliquots of the binding mixture to polyethylene microtubes (Sarstedt, 400 μl) containing 200 μl dibutyl phthalate and mineral oil (9/1, v/v) (modification of Gladhaug and Christoffersen, 1987). The samples were centrifuged in a Biofuge B microcentrifuge (Scientific Products) at 12,000*g* for 5 min to separate the cells from the reaction mixture. After cutting the cell pellet away from the supernatant, samples were counted in a gamma counter (Packard). Centrifugation of labeled ligand in the absence of cells indicated that less than 0.01% of the free ^{125}I-PB1 partitioned into the oil layer. Dissociation of ^{125}I-PB1 from cells was determined by first performing equilibrium binding at 4°C and then quickly pelleting and resuspending the cells in binding medium (dilution) either with or without 200-fold excess unlabeled PB1. Samples were next incubated at either 4° or 37°C, and aliquots were processed at increasing time intervals as described above.

Internalization assays

Internalization of the FnR was studied by three different techniques.

Acid/salt stripping. In the first method, internalization was determined by performing saturation binding with ^{125}I-PB1 at 4°C as described above. Samples were either then a) warmed to 37°C, or b) first excess antibody washed away and then they were warmed to 37°C. At designated time intervals, aliquots were removed, and the intracellular levels of PB1 were quantitated by a modification of the acid/salt elution method of Haigler et al. (1980). Briefly, the aliquots were mixed with an equal volume of 0.4 M acetic acid and 1 M NaCl (resulting pH of 3.0). The mixture was allowed to sit on ice at 4°C for 8 min and then was layered onto 200 μl of dibutyl phthlate/mineral oil and centrifuged. The cell-associated radioactivity resistant to the acid treatment was assumed to represent internalized ligand. Acid/salt treatment of cells maintained at 4°C after saturation binding indicated that this method is effective in removing >90% of the membrane-bound ligand.

Pharmacologic inhibition of recycling. Internalization of the FnR was also determined indirectly by treatment of CHO cells with the carboxylic ionophore monensin or the acidotropic amine chloroquine. Both of these compounds have been shown to inhibit recycling of endocytosed cell surface proteins; thus, the disappearance of receptors from the cell surface in their presence provides indirect evidence of internalization of the protein (Basu et al., 1981; Gladhaug and Christ-

offersen, 1988; Berg et al., 1983; Harford et al., 1983a,b; Maxfield, 1982; Van Leuven et al., 1980; Gonalez-Noriega et al., 1980). Cells in binding medium were treated at 37°C with 25 μM monensin, 10 μM chloroquine, 10 μg/ml cycloheximide, or 10 μg/ml tunicamycin. At increasing time intervals, aliquots were removed and placed on ice for 20 min. Saturation binding of ^{125}I-PB1 was performed and compared with untreated cells and cells maintained at 4°C. Using trypan blue exclusion and cell number as indicators, cells maintained in the various drugs at the given concentrations for up to 5 h at 37°C were 92–96% viable.

Surface labeling and immunoprecipitation. Alternatively, we followed internalization of the FnR by a variation of an immunoprecipitation procedure used by Vega and Strominger (1989) in their work with HLA class I antigens. CHO cells were surface-labeled with ^{125}I at 4°C. After being warmed to 37°C for various times, intact cells (1×10^7) were incubated on ice with 20 μg PB1 for 90 min in binding medium. Excess antibody was then removed by washing the cells three times in binding medium, and the cells were lysed for 1 h on ice in 200 μl lysis buffer (75 mM n-octyl-β-D-glucopyranoside in 10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM diisopropyl fluorophosphate, and 3 mM iodoacetamide). The lysate was cleared by centrifugation and then incubated with 200 μg Protein A Sepharose that had been equilibrated with 15 μg of rabbit anti-mouse IgG (RAM) and then washed with lysis buffer. After 1 h, the matrix was pelleted and washed four times with lysis buffer, and bound material was eluted by boiling with SDS sample buffer for 3 min. To be sure all the PB1-FnR complex had been removed from the lysate, the samples were re-incubated with Protein A Sepharose-RAM. After this second treatment, remaining labeled FnR (i.e., that which was inside the cell) was recovered by adding PB1 to the lysate for 90 min and then precipitating the complex with Protein A Sepharose-RAM as described above.

For some immunoprecipitations of total FnR, surface-labeled cells were lysed, and the lysate was cleared by centrifugation and incubated overnight at 4°C with PB1-Affi-gel or 7E2-Affi-gel. After the matrix was washed, FnR was eluted by boiling with SDS sample buffer for 3 min. All immunoprecipitants were electrophoresed on non-reducing, 7.5% polyacrylamide gels by the method of Laemmli (1970). Gels were dried, and autoradiography was performed using Cronex film (DuPont) and intensifying screens at −80°C, generally for 18 h.

RESULTS

Monoclonal PB1 specificity and binding to CHO cells

Specificity. Brown and Juliano (1985, 1988) have shown that the monoclonal antibody PB1 is specific for the intact FnR and is capable of inhibiting CHO cell adhesion to fibronectin-coated substrata; the monoclonal antibody 7E2 displays specificity for an epitope of the β_1 subunit and does not interfere with Fn-mediated cell adhesion. Immunoprecipitation with PB1 results in recovery of the characteristic FnR α-β heterodimer; repetitive immunoprecipitation with this antibody can effectively deplete this integrin from a heterogenous sample. Subsequent treatment of the FnR-depleted lysate with 7E2-Affi-gel precipitates at least 1 other β_1 integrin, which has an α subunit mobility slightly faster than that of the α_5 polypeptide (Sczekan and Juliano, unpublished observations).

Binding characteristics. Figure 1A shows the specific binding of ^{125}I-PB1 to CHO cells at 4°C. Saturation is reached at about 1.5 μg/ml PB1 (about 10 nM) with around 200 fmol bound by 10^6 cells. A Scatchard analysis of the data (Fig. 1A, inset) reveals a single PB1 binding site with an affinity of about 4.2×10^8 M; there are approximately 1.4×10^5 binding sites per cell. These results are in accord with values obtained earlier using fixed cells (Brown and Juliano, 1986).

A comparison of the time course of PB1 binding to CHO cells at 37°C and 4°C was also made (Fig. 1B). At a saturating concentration of ^{125}I-PB1 (2.5 μg/ml), binding at 37°C reaches a peak value of around 220 fmol/10^6 cells in about 15 min and drops to less than 25% of this value in 2 h. This suggests that antibody-receptor complexes may be shed from the cells at 37°C. Binding of ^{125}I-PB1 to CHO cells at 4°C reaches a maximum of about 200 fmol/10^6 cells in 90 min and varies less than 10% from this amount over the next 3 h. An approximation of the initial rates of binding is seen in Figure 1C. At 37°C about 13.5 fmol min^{-1} are initially bound by 10^6 cells, while the rate drops to 4.6 fmol min^{-1} at 4°C. Therefore, while the binding kinetics are slower at 4°C than at 37°C, the maximal amount of PB1 bound is very similar.

The reversibility of PB1 binding to CHO cells was examined in dissociation studies (Fig. 2). After allowing ^{125}I-PB1 to bind to cells at 4°C to about 85% of their capacity, the cells were quickly pelleted and resuspended in binding medium with or without 200 μg/ml unlabeled PB1. Aliquots were either maintained at 4°C or moved to 37°C, and the amount of ^{125}I-PB1 bound to the cells was monitored as a function of time. Under these conditions, where the further association of the radio-labeled ligand (PB1) with the FnR is negligible,

$$d[PB1 - FnR]/dt = -k_2[PB1 - FnR]$$

where k_2 is the dissociation rate constant with units of min^{-1} and [PB1 − FnR] represents the concentration of ^{125}I-PB1 specifically bound to the FnR. Therefore, a plot of $\log_{10}[bound]_t/[bound]_0$ (concentration PB1 bound at any time, t, divided by concentration of PB1 bound at time 0) versus time yields a linear transformation with a slope of $-2.303\ k_2$. Figure 2B shows this transformation; the linearity ($r = -0.97$ to -0.99) of the plots is consistent with what is expected for the dissociation of a single bimolecular complex. The variable results at 37°C (in the presence and absence of excess PB1) may be indicative of negative cooperativity among the sites (Limbird, 1986); alternatively, the apparently rapid dissociation in the presence of excess antibody at 37°C may reflect shedding of antibody-receptor complexes from the cell. The apparent dissociation rate constants ranged from 0.0212 min^{-1} to 0.114 min^{-1}.

Using these k_2 values and the K_d calculated from the Scatchard analysis (Fig. 1A), association rate constants, k_1, were estimated using

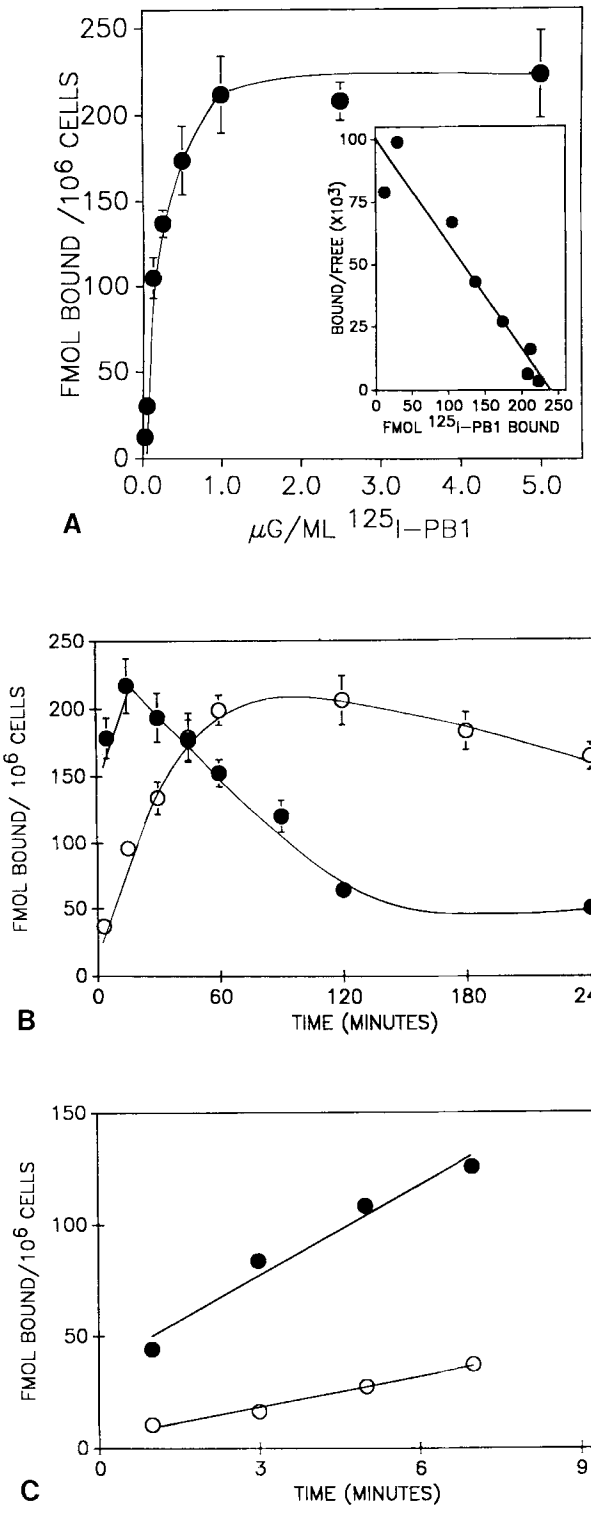

Fig. 1. Specific binding of monoclonal antibody PB1 to CHO cells. **A:** Binding of ^{125}I-PB1 to CHO cells in suspension measured as a function of antibody concentration after 90 min incubation at 4°C. Data shown are the mean of two experiments performed in triplicate. **Inset:** Scatchard analysis of the data in part A. **B:** Comparison of the specific binding of ^{125}I-PB1 to CHO cells at 4°C and 37°C as a function of time. Cells in suspension were incubated with 2.5 μg/ml ^{125}I-PB1 at 4°C(○-○) or 37°C (●-●). **C:** The initial rates of ^{125}I-PB1 binding to CHO cells at 4°C (○-○) and 37°C (●-●).

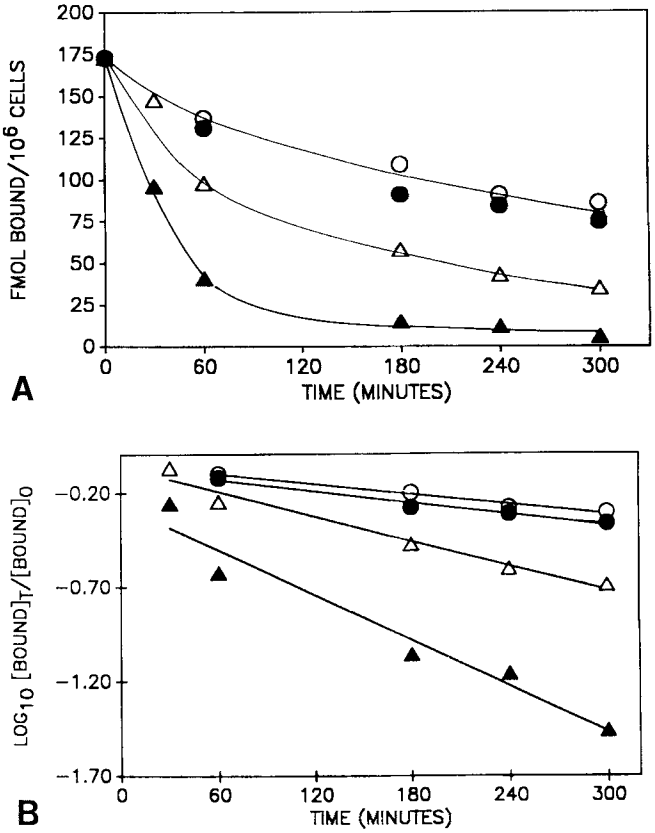

Fig. 2. Reversibility of PB1 binding to CHO cells. **A:** After saturation binding of ^{125}I-PB1 to CHO cells at 4°C, the dissociation of the antibody from the cells was followed as a function of time and temperature in the presence or absence of excess unlabeled PB1. 4°C: ○-○, without excess PB1; ●-●, with excess PB1. 37°C: △-△, without excess PB1; ▲-▲, with excess PB1. **B:** A linear transformation of the data in (A) for calculation of the dissociation rate constant, k_2, as described in the text.

$$K_d = k_2(min^{-1})/k_1(M^{-1}min^{-1})$$

These values varied from 8.8×10^6 $M^{-1}min^{-1}$ at 37°C to 4.8×10^7 $M^{-1}min^{-1}$ at 4°C. The association and dissociation rate constants estimated for the PB1-FnR interaction are in the range expected for the reaction kinetics of antibody binding to an antigen on the cell surface (Mason and Williams, 1980).

We also monitored the degradation of PB1 by measuring the TCA-precipitable counts in the cells and the supernatant at various time points (Table 1). Little or no degradation was evident for up to 5 h at either 4°C or 37°C.

Internalization of the CHO cell fibronectin receptor

Internalization of ligated FnR. Figure 3 shows the results of the internalization of ^{125}PB1 by CHO cells as determined by the acid/salt elution technique. Cells were saturated at 4°C with ^{125}I-PB1 and then incubated at 37°C in the presence or absence of excess ^{125}I-PB1. After membrane-bound ligand was removed by acid/salt stripping, internalized ^{125}I-PB1 was quantitated and is expressed as a percentage of the total ^{125}I-PB1 bound by the cells prior to the acid stripping (t = 0). In both cases, approximately 30% or 36,000 mole-

TABLE 1. Percent TCA-precipitable counts associated with the cells or the supernatant following equilibrium binding of ^{125}I-PB1 presented as a function of time and temperature of incubation

	Cell-associated		Supernatant	
Time	4°C	37°C	4°C	37°C
0	93.2	93.0	86.3	86.2
0.5 h	93.2	92.9	86.1	85.1
2.0 h	93.0	92.8	86.0	85.0
5.0 h	92.9	93.0	86.1	85.2

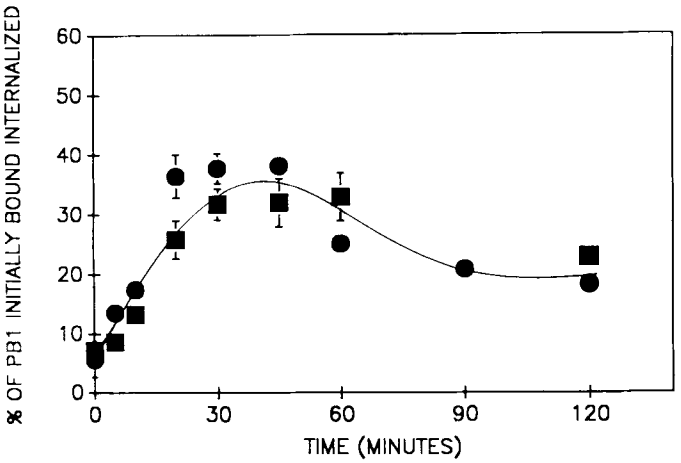

Fig. 3. Internalization of ligated FnR. CHO cells were saturated with ^{125}I-PB1 at 4°C (2.5 μg/ml antibody, 90 min) and then incubated at 37°C in the presence (●-●) or absence (■-■) of excess ^{125}I-PB1. Internalized material was measured by the acid/salt elution technique as described in "Materials and Methods" and is expressed as a percentage of the total amount of ^{125}I-PB1 initially bound by the cells at 4°C. The results are the mean of three experiments performed in triplicate.

cules/cell (after correction for residual ligand not removed by the acid/salt elution) are internalized by the cells. This occurs with a $t_{1/2}$ of about 7 min, a time comparable to that of other endocytosed cell surface receptors (Steinman et al., 1983). A peak is reached around 15 min, and there is a decline in the amount of antibody accumulated intracellularly starting about 60 min; the decline may represent a modest amount of degradation followed by excretion of degraded antibody.

Internalization of the non-ligated FnR: effect of chloroquine and monensin on the cell surface receptor pool. Constitutive endocytosis of the FnR was studied by observing the effects of monensin and chloroquine on the cell surface FnR pool (Fig. 4). After treatment at 37°C with 25 μM monensin or 10 μM chloroquine for the times indicated, the cells were equilibrated to 4°C, and saturation binding with ^{125}I-PB1 performed. The amount of ^{125}I-PB1 bound by the treated cells was compared to that of cells maintained at 4°C and to cells maintained at 37°C but receiving no drug treatment. By 90 min the number of PB1 binding sites on the surface of the cells treated with chloroquine or monensin had dropped 30% from that of control values. Treatment of cells with 10 μg/ml cycloheximide or 10 μg/ml tunicamycin had no effect on the amount of PB1 bound (not shown). Also, treatment of

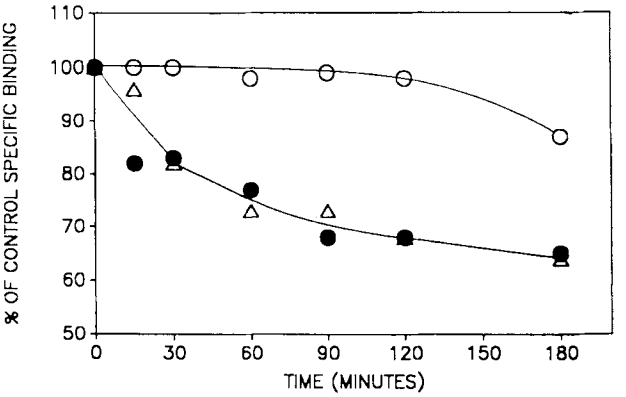

Fig. 4. The effect of monensin and chloroquine on the cell surface FnR pool. CHO cells were treated with 25 μM monensin (△) or 10 μM chloroquine (●) at 37°C for the times indicated. After chilling to 4°C, the amount of ^{125}I-PB1 bound by the treated cells was compared to untreated cells (○). The results are expressed as a percentage of the total ^{125}I-PB1 specifically bound by untreated cells.

cells with monensin or chloroquine at 4°C had no effect on the kinetics of binding or the amount of PB1 bound (not shown). Therefore, comparing Figures 3 and 4, it appears that about 30% of the total FnR population is available for internalization regardless of the state of ligand occupation.

Qualitative examination of FnR internalization by immunoprecipitation. Physical evidence that the FnR is in fact being internalized was obtained by selective immunoprecipitation of membrane-bound and internal FnR; these results are seen in Figure 5. After surface-iodination at 4°C, cells were moved to 37°C to initiate internalization. The relative amounts of labeled FnR on the inside and outside of the cells were followed as a function of time by immunoprecipitation with PB1. Within 30 min of incubation, the cells have accumulated detectable amounts of the FnR intracellularly; there appears to be very little increase between 30 min and 2 h. There is certainly a decrease in the amount of FnR precipitated from the surface of the cells, although precise quantitation by this technique is difficult. The slow decrease seen between 30 min and 2 h is consistent with the loss of antibody binding seen in Figures 1 and 2A and may be partially due to shedding of FnR. These results provide evidence that the FnR is in fact being internalized by the cells in the absence of a ligand and that the loss of PB1 binding sites seen in Figure 4 is not due to degradation of the receptor.

DISCUSSION

We have presented data that suggest that a portion of the CHO FnR population is internalized in a constitutive manner. First we demonstrated the endocytosis of approximately 30% of the cell surface FnR population by following the internalization of the receptor-specific antibody ^{125}I-PB1 upon warming cells to 37°C. This process occurred with a $t_{1/2}$ of about 7 min, which is comparable to that of many other cell surface receptors (Steinman et al., 1983). Why only this moderate percentage of the total is internalized is unclear. A similar situation was observed by Vega and Stro-

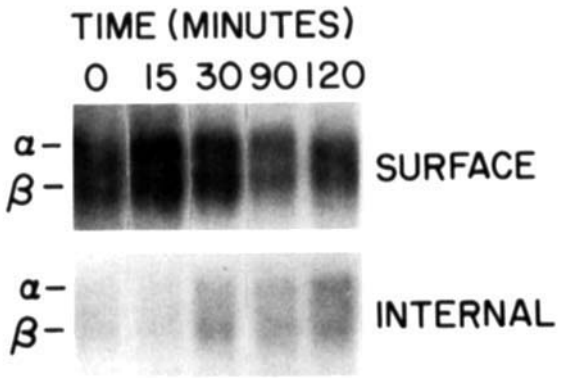

Fig. 5. Selective immunoprecipitation of cell surface and internalized FnR. CHO cells were surface-labeled with ^{125}I at 4°C. After washing, the cells were warmed to 37°C and at the indicated times, aliquots (1×10^7 cells) were chilled to 4°C. The relative amounts of ^{125}I-FnR on the cell surface and inside the cells were then assayed as described in the text. **Upper row:** ^{125}I-FnR remaining on the cell surface as a function of time at 37°C. **Lower row:** ^{125}I-FnR internalized by the cells as a function of time at 37°C. The α and β subunits are indicated.

minger (1989) with HLA class I antigens. They discussed 3 possibilities for this phenomenon that would also be relevant in our case: 1) the percentage of the total receptor internalized is a reflection of the recycling dynamics of the receptor; 2) there is a pool of receptors physically unavailable for internalization; or 3) there is a chemically distinct pool of receptors unavailable for internalization.

We next showed that the non-ligated FnR is internalized constitutively, that is, movement of the receptor to the inside of the cell occurred in the absence of a ligand. This was determined by monitoring the decrease in the number of cell surface receptors in the presence of monensin or chloroquine, reagents known to disrupt receptor recycling (Mellman et al., 1986). Several significant points are to be derived from this set of experiments. First, we once again observed that only about 30% of the total FnR population was active in the internalization process. This finding strengthens the idea of either a physically or chemically distinct class of receptors available for turnover. Second, the pool of cycling receptors measured by this method was similar to that observed with ^{125}I-antibody bound to the receptor, thus the monoclonal antibody PB1 which we used as a ligand does not seem to induce or enhance endocytosis. Third, the decrease in cell surface receptor number seen with chloroquine or monensin treatment is probably not due to the prevention of newly synthesized material from reaching the cell surface since cycloheximide and tunicamycin have no effect on the FnR number within the time limits of these experiments. In short, our findings suggest a constitutive internalization process involving about 30% of the total receptor pool.

Additional support for this concept was obtained by surface labeling cells at 4°C and then selectively immunoprecipitating surface and intracellular FnR after warming to 37°C. A portion of the radio-labeled receptor on the cell surface is in fact internalized from 0–30 min after warming, and the amount accumulated intracellularly does not seem to increase significantly between 30 min and 2 h. This indicates once again that only a portion of the total FnR pool is internalized.

While there remains the possibility that the large, residual FnR population is endocytosed but at a very slow rate, we are left with the question of the difference in these two pools of FnR. To our knowledge, there is no evidence for chemically distinct populations of fibronectin receptor in the CHO cell line. Alternatively, our results could be explained by actual physical restraints placed on FnR movement into coated pits for internalization. Duband et al. (1988) have measured the lateral mobility of the avian FnR (CSAT) in cells that were in varying states of locomotion. The percent mobile fraction in locomoting cells was found to be about 80% at 37°C, while the percent mobile receptor in the focal contacts, and fibrillary streaks of intermediate mobility and stationary cells was around 18%. In cells of intermediate motility, the mobile fraction of receptors outside the focal contacts and fibrillary streaks was around 30%. Whether this reduced mobility is a reflection of interaction of the receptor with the cytoskeleton or its lateral association with other membrane components remains to be determined. In either case, such restraints might imply that not all receptors are able to move to the coated pits for endocytosis.

While this manuscript was in preparation, Bretscher, (1989) utilizing a quite different set of techniques, reported that about 14% of the FnR is internal at any given time. He has related his findings to a scheme proposing that unoccupied receptors anywhere on the cell surface can be endocytosed and transported to the leading edge of a spreading or moving cell. While our results also show that the FnR is constitutively internalized, it appears that not all receptors are available for this type of recycling.

Although our primary interest in this report is with the internalization of FnR, our data also indicate the existence of a slower process in which a portion of antibody-ligated receptor is shed or lost from the cell. Thus, Figure 1B shows a large reduction in the total amount of ^{125}I-PB1 bound after 60 min or more of incubation at 37°C. This cannot be due to internalization followed by degradation since only a moderate (30%) fraction of the receptor is internalized (Fig. 3) and there is no evidence of proteolytic degradation of the antibody in the cells or supernatant during this period (Table 1). This suggests either that the receptor is proteolyzed at the cell surface leading to loss of antibody binding or that fragments of membrane enriched in antibody-receptor complexes are shed from the cell. We think the latter alternative is more likely since the hamster FnR is quite resistant to proteolysis (Sczekan and Juliano, unpublished results). Thus, while FnR internalization seems to be constitutive, the overall surface display of FnR can be modulated by exposure to antibody, probably through shedding of the cell surface antigen. It remains to be seen whether the 30% of FnR that is rapidly internalized at 37°C and the approximately 70% of the FnR that is more slowly (3 h) lost from the cell represent distinct receptor populations.

In summary, we have shown that about 30% of the CHO cell FnR population is available for constitutive internalization, while the remainder of the FnR is not rapidly internalized. The difference in these 2 appar-

ently disparate pools of receptor remains to be determined, and its elucidation will hinge on a better characterization of integrin association with membrane and cytoskeletal components.

ACKNOWLEDGMENTS

This work was supported by NIH grant GM26165 to R.L. Juliano.

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