Fc Receptor Endocytosis Is Controlled by a Cytoplasmic Domain Determinant That Actively Prevents Coated Pit Localization

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Abstract. Macrophages and B-lymphocytes express two major isoforms of Fc receptor (FcRII-B2 and FcRII-B1) that exhibit distinct capacities for endocytosis. This difference in function reflects the presence of an in-frame insertion of 47 amino acids in the cytoplasmic domain of the lymphocyte isoform (FcRII-B1) due to alternative mRNA splicing. By expressing wild type and mutant FcRII cDNAs in fibroblasts, we have now examined the mechanism by which the insertion acts to prevent coated pit localization and endocytosis. We first identified the region of the FcRII-B2 cytoplasmic domain that is required for rapid internalization. Using a biochemical assay for endocytosis and an immuno-EM assay to determine coated pit localization directly, we found that the distal half of the cytoplasmic domain, particularly a region including residues 18–31, as needed for coated pit-mediated endocytosis. Elimination of the tyrosine residues at position 26 and 43, separately or together, had little effect on coated pit localization and a partial effect on endocytosis of ligand. Since the FcRII-B1 insertion occurs in the membrane-proximal region of the cytoplasmic domain (residue 6) not required for internalization, it is unlikely to act by physically disrupting the coated pit localization determinant. In fact, the insertion was found to prevent endocytosis irrespective of its position in the cytoplasmic tail and appeared to selectively exclude the receptor from coated regions. Moreover, receptors bearing the insertion exhibited a temperature- and ligand-dependent association with a detergent-insoluble fraction and with actin filaments, perhaps in part explaining the inability of FcRII-B1 to enter coated pits.

Fc receptors for IgG (FcR)[1] are members of a large multigene family that mediates diverse functions critical to the cellular immune response in various cell types (Mellman, 1989; Ravetch and Kinet, 1991). One of the best characterized of these receptors is the product of the murine FcRII gene. This receptor is specific for multivalent IgG-antigen complexes and is expressed as two major isoforms, FcRII-B1 and -B2, in macrophages and lymphocytes, respectively (Hibbs et al., 1986; Lewis et al., 1986; Ravetch et al., 1986). The two receptors have identical extracellular and membrane spanning domains, but differ in their cytoplasmic tails due to cell-type specific alternative mRNA splicing (Qiu et al., 1990). While the macrophage FcRII-B2 isoform has a cytoplasmic domain of only 47 amino acids, the B-cell FcRII-B1 isoform contains an in-frame insertion of 47 additional amino acids. The insert occurs at a site six residues from the membrane.

By stably transfecting the two FcRII isoforms into receptor-negative fibroblasts, we found that these variations in the cytoplasmic domain are associated with functional differences between the two receptors (Miettinen et al., 1989). While FcRII-B2 was found to mediate the efficient endocytosis and delivery to lysosomes of ligand-receptor complexes via clathrin-coated pits, FcRII-B1 was almost completely deficient at coated pit localization and mediated ligand internalization even less efficiently than a tail-minus FcRII mutant. This difference also appears to correlate with the relative abilities of endogenous FcRII in macrophages and B-lymphocytes to mediate the endocytosis of IgG-complexes (Amigorena, S., C. Bonnerot, D. Choquet, G. Raposo, J.-G. Guillet, C. Sautes, W. H. Fridman, J. Drake, W. Hunziker, P. Webster, and I. Mellman, manuscript submitted for publication; Mellman and Plutner, 1984; Miettinen et al., 1989). Thus, the cytoplasmic tail insertion in FcRII-B1 somehow acts to disrupt the receptor's ability to localize in coated pits. The insert also introduces several serine phosphorylation sites that are used in conjunction with FcRII-B1-mediated regulation of B-cell activation (Hunziker et al., 1990). Phagocytosis, however, does not seem to require a functional coated pit localization domain since both FcRII-B1 and FcRII-B2 were able to mediate internalization of IgG-coated parasites in transfected CHO cells (Joiner et al., 1990).

To understand how alternative mRNA splicing regulates Fc receptor-mediated endocytosis, it is first necessary to identify what region or regions of the FcRII-B2 cytoplasmic tail are required for coated pit localization. Most plasma membrane receptors studied thus far have been shown to selectively accumulate in coated pits due to the presence of a tyrosine-containing recognition determinant (Alvarez et

1. *Abbreviation used in this paper*: FcR, Fc receptor.

al., 1990; Davis et al., 1987; Jing et al., 1990; Lazarovits, 1988; Lobel et al., 1989; McGraw and Maxfield, 1990). The FcRII-B2 cytoplasmic tail contains two tyrosine residues, the sequence surrounding one of which at least superficially resembles sequences found near the critical tyrosines of other coated pit receptors (Ktistakis et al., 1990). This tyrosine also falls within a region of high homology between the murine and human receptors (Brooks et al., 1989; Stengelin et al., 1988; Stuart et al., 1987, 1989), further suggesting its functional importance. We now show that although the homologous region is at least one element of the FcRII-B2 cytoplasmic tail that is required for rapid endocytosis, neither of the two tyrosine residues are essential for coated pit localization and only partially required for internalization. Moreover, our data suggest that the cytoplasmic domain insertion characteristic of FcRII-B1 does not block endocytosis by disrupting the coated pit localization domain, but actively prevents coated pit localization, at least in part, by tethering the receptor to the cytoskeleton.

Materials and Methods

Transient Expression in COS-1 Cells

Cells were grown in monolayer cultures in DME containing 5% FBS. One day before transfection cells were plated at a density of 5×10^5 cells/35 mm well. Cells were transfected using the DEAE-dextran method (Adams and Rose, 1985) with either pJC119R (Miettinen et al., 1989; Sprague et al., 1983), or SRα (Takebe et al., 1988) as expression vector. The FcR cDNAs were ligated into the unique EcoRI site of respective vector. All experiments were performed 18–24 h after the transfection.

Stable Expression in CHO Cells

Permanent FcRII-expressing CHO cell lines were produced after transfection using the amplifiable expression vector pFRSV as described previously (Miettinen et al., 1989). Transfected cells were maintained as monolayer cultures in a selection medium containing 10–100 μM methotrexate and 4 μg/ml folic acid in α-MEM supplemented with 5% FBS. After the cells had reached a stable growth rate in 10 μM methotrexate, 3–5% of the highest expressing cells were collected using a fluorescence-activated cell sorter (FACS Star, Becton Dickinson Microbiology Sys., Cockeysville, MD) as follows: cells grown in suspension culture were incubated at a density of 2×10^5 cells/ml at 0°C for 90 min with 20 μg/ml 2.4G2 IgG, a rat mAb against FcRII; the cells were washed twice with α-MEM containing 8% FBS and incubated at 0°C for 30 min with fluorescein-conjugated rabbit anti-rat IgG; after washing, the cells were resuspended in PBS at 2×10^5 cells/ml and finally sorted under sterile conditions using window settings of 3.8 or 4.25 log units, depending on expression level. Nontransfected CHO cells were used as negative controls.

Site-directed Mutagenesis and Polymerase Chain Reaction

The FcRII-B2 and FcRII-B1 cDNAs were cloned in the T7 orientation to the EcoRI site of Bluescript SK$^+$. Single stranded template was prepared using M13 K07 helper phage. In vitro mutagenesis was performed as described using the Bio-Rad mutagenesis kit (Bio-Rad Laboratories, Cambridge, MA) (Kunkel et al., 1987). The following synthetic oligonucleotides were used as primers to generate the mutations. The oligonucleotides 5'-T ACG ATC ACC GCT TCA CTT CTC A-3' and 5'-CA GAG CAT GAT GCG CAG AAC CAC ATT TAG-3' were designed to change the FcRII-B2 cytoplasmic tail tyrosines in positions 26 (TAC) and 43 (TAC), respectively, to alanines (GCT and GCG) (Fig. 1). The double tyrosine mutant in B2 was generated by making a single-stranded template from the B2 cDNA containing the mutation in position 26 and then priming with the second oligonucleotide primer. An MluI restriction site was generated just before the stop codon in the wild type FcRII-B2 cDNA by using 5'-GAT TAC CAG AAC ACG CGT TAG TCT CCC TTG-3' as primer. This mutation changed the two last amino acids, histidine and isoleucine, to threonine and arginine. All mutations were transformed into *Escherichia coli* C600 and the mutations were confirmed by sequencing using the Sanger dideoxy method (Sanger et al., 1977). To ensure that no other mutations had been introduced during the in vitro mutagenesis, the entire transmembrane and cytoplasmic domains of respective constructs were also sequenced.

The construction of the FcRII-B2 tail-minus deletion mutant has been described elsewhere (Miettinen et al., 1989). The FcRII-B2 CT31 and CT18 deletion mutants were constructed essentially according to the Stratagene manual. An EcoRI/HincII fragment of the FcRII-B2 cDNA was cloned into Bluescript KS$^+$ in T7 orientation. The plasmid was linearized with HincII and KpnI and digested first 1–5 min at 23°C with Exonuclease III followed by a 30-min digestion at 37°C with mung bean nuclease. The overhangs were filled in, and the plasmid was religated using an XbaI stop codon linker. Clones were screened by digestion of minipreps with XbaI and the clones of interest were identified by sequencing. In both CT31 and CT18 the last amino acid (Thr18 and His31) was preserved.

The FcRII-B1 insert cDNA with overhanging MluI restriction sites (151 bp) was synthesized by polymerase chain reaction (Saiki et al., 1988) using the following pair of primers: 5'-C ACT ACG CGT GCT CTC CCA GGA AAC-3' and 5'-A CTG ACG CGT GTA TGG GCT GCT GCT TTG-3' (MluI-site underlined). The reaction was done using a PCR-kit (Perkin Elmer Corp., Norwalk, CT) and a cycler (Techne Inc., Princeton, NJ).

Fluorescence Microscopy

Cells on coverslips were incubated for 2 h at 0°C with 20 μg/ml IgG complexes. The complexes, consisting of rabbit anti-2,4-DNP IgG and DNP-modified BSA (DNP-BSA) combined at a molar ratio of 2.5:1, were formed as previously described (Mellman and Plutner, 1984). After washing, the cells were incubated for 30 min at 37°C, fixed, and prepared for immunofluorescence essentially as previously described (Miettinen et al., 1989). To visualize F-actin, cells were incubated after fixation and permeabilization with rhodamine-conjugated phalloidin (Molecular Probes Inc., Eugene, OR).

Endocytosis Assay

Cells were grown to ~70% confluency in 24-well plastic dishes and incubated for 20 min on ice and 30 min at 37°C before the experiment to maximize cell spreading. HRP-IgG complexes were formed at 37°C for 30 min using 21 μg/ml affinity-purified rabbit anti-HRP IgG and 5 μg/ml HRP (Sigma Chemical Co., St. Louis, MO). Cells were rinsed once with ice-cold binding buffer (PBS containing 0.2% BSA, 1 mg/ml glucose, and 1 mM sodium pyruvate) and subsequently labeled with HRP-IgG complexes diluted 1:20 with binding buffer. After exactly 45 min on ice, the cells were washed four times with binding buffer and then incubated at 37°C in prewarmed binding buffer for various times. The cells were then cooled on ice and rinsed twice with PBS. 200 μl PBS $\pm$ 0.25% Triton X-100 was then added and incubated for 10 min on ice. 500 μl assay buffer (PBS containing 0.75 mg/ml *o*-phenylenediamine dihydrochloride and 0.023% hydrogen peroxide) was then added to detect total (+ detergent sample) vs. cell surface-bound (− detergent sample) HRP. The samples were incubated for 20 min (during which time the reaction remained linear) and then stopped by adding 300 μl 1 M HCl. 200 μl aliquots were transferred to a 96-well ELISA plate and the absorbance at 492 nm measured using a Titertek Multiscan plate reader (Flow Labs, McLean, VA). The amount of internalized ligand was determined by calculating the difference in absorbance measured with and without Triton X-100 as described previously (Drake et al., 1989).

Immunogold Labeling and EM

Gold sols (7–9 nm) were made by tannic acid-citrate reduction of gold chloride (Slot and Geuze, 1985). The gold-DNP/BSA complexes were formed and purified as described (Miettinen et al., 1989; Ukkonen et al., 1986). Subconfluent CHO monolayers in 22-mm wells were incubated with 20 μg/ml gold-IgG complexes for 2 h at 0°C. Cells were washed with cold PBS and dipped into prewarmed medium for 1.5 min. After the brief incubation at 37°C the cells were dipped into cold PBS and processed for EM as previously described (Ukkonen et al., 1986). Random micrographs of cells were taken at 10,000× with a JEM-100 CX electron microscope. Gold particles were counted from micrographs and in some cases (B2 A26, B2/B1-R, and B2/B1-W) directly from the screen. For each cell line, the gold particles were counted from a total of at least 0.75 mm of cell surface contour length determined using a morphometer (Carl Zeiss, Inc., Thornwood, NY). The data shown for the coated pit localization of FcRII-B1, B2, and -tail-minus are in part from a previous publication (Miettinen et al., 1989).

Triton Extractions and Immunoprecipitations

A subconfluent monolayer of cells on a 6-cm dish was labeled overnight with 0.4 mCi/ml [^{35}S]-cysteine in cysteine-free α-MEM containing 8% dialyzed heat-inactivated FBS and 10 mM Hepes, pH 7.2. The labeling was terminated by rinsing the cells with PBS and the cells were chased for 1 h in complete α-MEM containing 8% heat-inactivated FBS. The cells were then incubated for 30 min with or without ligand (20 μg/ml IgG-complex or rabbit anti-FcR serum 1:100) on ice or at 37°C. After rinsing with cold PBS the cells were extracted for 10 min at 0°C in 1 ml "cytoskeleton buffer" (CSK) (0.5% Triton X-100, 10 mM K-PIPES [pH 6.8], 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, and 1 mM PMSF) as described by Fey et al. (1984). The plates were scraped using a rubber policeperson and the soluble and insoluble fractions were separated by a 1-min spin in a centrifuge (Eppendorf Inc., Freemont, CA). The Triton X-100 insoluble fraction was solubilized in 100 μl buffer containing 1% SDS, 50 mM Tris-HCL (pH 8.8), 5 mM EDTA, and 1 mM PMSF, and the volume was adjusted to 1 ml with CSK. After adding SDS to the soluble fraction (0.1% final concentration), both fractions were immunoprecipitated using 3 μl rabbit anti-FcR serum (2 h, 4°C) followed by 10 μl of a 10% (wt/vol) suspension of fixed *Staphylococcus aureus* cells (Zysorbin, Zymed Labs. Inc., South San Francisco, CA) that had been previously washed in PBS containing 1 mg/ml BSA and 0.5% Triton X-100. After precipitation, the cells were washed with RIPA buffer (10 mM Tris-HCl, pH 7.4, 0.1% SDS, 1% deoxycholate, 1% NP-40, and 0.15 M NaCl) and resuspended in 5 μl buffer containing 0.2 M sodium phosphate (pH 8.2), 1% SDS, and 1% mercaptoethanol. The samples were denatured by heating to 100°C for 3 min, and NP-40 in 0.2 M sodium phosphate (pH 8.2) was added to give a final concentration of 0.2% SDS and 1.4% NP-40. Samples were incubated for 16 h at 37°C with 0.15–0.2 units recombinant *N*-glycanase (Genzyme Corp., Cambridge, MA), mixed with an equal volume of 2× electrophoresis sample buffer, heated to 100°C for 3 min and run on a 15% SDS-polyacrylamide gel. After staining and destaining, the gel was soaked in 0.2 M sodium salicylate (pH 7.2), dried, and exposed on Kodak X-Omat film (Eastman Kodak Co., Rochester, NY) at −75°C. After developing the X-ray film, the intensity of the bands was quantified using an image processor (Kodak Visage 2000, Eastman Kodak Co.) supported by a Sun Microsystems workstation.

Results

A Conserved Tyrosine-containing Region of the Fc Receptor Cytoplasmic Tail Is Required for Efficient Endocytosis

We have previously shown that efficient endocytosis of ligand by FcRII-B2 requires the receptor's cytoplasmic domain, since a tail-minus deletion mutant was internalized with reduced efficiency and localized in coated pits with a 10–15-fold lower frequency than the wild type receptor (Miettinen et al., 1989). To examine the region(s) of the cytoplasmic domain that are required for coated pit localization and rapid endocytosis, a series of deletion mutations was made. Two of these deletion mutants, CT31 and CT18, with 31 and 18 residues remaining of the 47 amino-acid cytoplasmic domain, respectively (Fig. 1), were inserted into an SV40-based expression vector for transient expression in COS-1 cells. One day after transfection, cells were incubated on ice with ligand consisting of rabbit anti-DNP IgG:DNP-BSA (IgG-complexes) and then shifted to 37°C for 30 min to allow endocytosis. Cells were then prepared for immunofluorescence microscopy. As shown in Fig. 2, cells transfected with the wild type FcRII-B2 had a vacuolar staining pattern with only some staining on the cell surface (Fig. 2 *A*). Similarly, cells expressing the CT31 deletion mutant which lacks the last 16 amino acids of the cytoplasmic tail, exhibited an intracellular staining pattern resembling that of FcRII-B2 (Fig. 2 *B*). In contrast, in cells expressing the CT18 mutant, most of the staining was found on the cell surface (Fig. 2 *C*), similar to the pattern exhibited by cells transfected with the tail-minus mutant (Miettinen et al., 1989). These results suggested that the domain for rapid endocytosis was at least partly located between amino acids 18 and 31. This region corresponds to the only stretch of conserved amino acids between FcRII-B2 and its human homologue hFcRII-C; the human and mouse receptors are 85% identical throughout this sequence (11 out of 13 amino acids), but less conserved (35%) elsewhere in the cytoplasmic tail.

Tyrosine Residues in the FcRII-B2 Tail Are Incompletely Required for Efficient Endocytosis

The cytoplasmic domain of FcRII-B2 contains two tyrosine residues, the first (at position 26 [Y26]) being in the highly conserved region required for rapid endocytosis. Y26 is also located within a sequence that may have properties similar to coated pit localization domains in other receptors (Ktistakis et al., 1990): a nonhelical structure containing charged and/or polar residues to the amino-terminal side of the tyrosine, and one or two charged or polar residues to the carboxy-terminal side. To examine whether Y26 was needed for coated pit localization and endocytosis, we changed it to an alanine (A26) (Fig. 1) and transfected the mutant receptor into COS-1 cells. Surprisingly, immunofluorescence indicated that Y26 residue was not necessary for rapid endocytosis. After 30 min, much of the ligand in cells transfected with the A26 mutant was in intracellular vesicles (Fig. 2 *D*) similar to cells expressing the FcRII-B2 wild type.

To determine whether the second tyrosine at position 43 (Y43) might instead be the critical tyrosine, we produced two additional mutants in which Y43 was changed to an alanine

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B1 wt      KKKQVPALPGNPDHREMGETLPEEVGEYRQPSGGSVPVSPGPPSGLEPTSSSPYNPPDLEEAAKTEAENTITYSLLKHPEALDEETEHDYQNHI
                    Tail-minus          CT18          CT31
                    ↓                   ↓      O      ↓          O
B2 wt      KKKQVPDNPPDLEEAAKTEAENTITYSLLKHPEALDEETEHDYQNHI

B2/B1-R    KKKQVPDNPPDLEEAAKTEAENTITYSLLKHPEALDEETEHDYQNTRALPGNPDHREMGETLPEEVGEYRQPSGGSVPVSPGPPSGLEPTSSSPYTR

B2/B1-W    KKKQVPDNPPDLEEAAKTEAENTITYSLLKHPEALDEETEHDYQNTRVWAAACWLQSRWRPWADRH
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Figure 1. Amino acid sequences of the cytoplasmic domains of wild type and mutant Fc receptors. The cytoplasmic domain sequences of FcRII-B1, -B2, and various mutants are shown in the single letter amino acid code. The underlined sequence in B1 shows the 47 amino acid insertion. The tyrosine residues of the B2 receptor in positions 26 and 43 are marked with open circles and the deletion mutants are marked with arrow heads. Additional or foreign amino acids in the B2/B1-R and B2/B1-W constructs are indicated in bold; the location of the B1 insert in B2/B1-R is underlined.

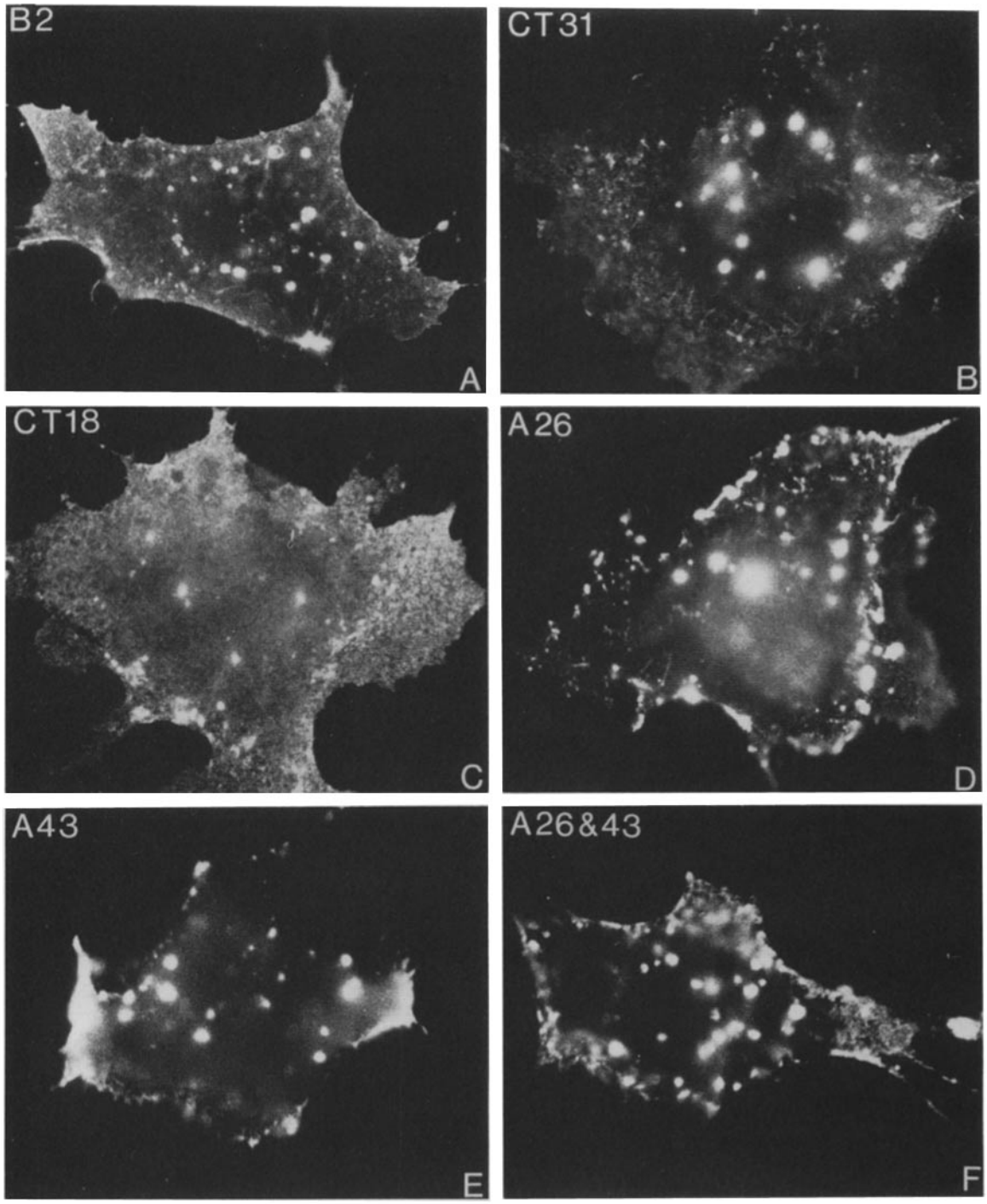

Figure 2. Internalization of IgG-complexes by COS-1 cells transiently expressing wild type or mutant FcRII-B2. Cos-1 cells were transfected with cDNAs encoding FcRII-B2 or the indicated FcRII-B2 mutants using the pJC119R expression vector (Miettinen et al., 1989; Sprague et al., 1983). 24 h later, cells were incubated with 20 μg/ml IgG complexes for 2 h at 0°C. After washing with PBS-glucose, the cells were warmed to 37°C for 30 min to allow internalization, fixed using paraformaldehyde-lysine-periodate, and permeabilized with saponin. To visualize both surface-bound and internalized ligand, the permeabilized cells were stained with affinity-purified $F(ab')_2$ fragments of a fluorescein-conjugated goat anti-rabbit IgG. (*A*) FcRII-B2; (*B*) FcRII-B2 CT31; (*C*) FcRII-BN2 CT18; (*D*) FcRII-B2 A26; (*E*) FcRII-B2 A43; (*F*) FcRII-B2 A26&43.

(A43) alone or together with Y26 (A26/43 double mutant). When expressed transiently in COS-1 cells, both mutants yielded staining patterns that were indistinguishable from wild type FcRII-B2 and A26 (Fig. 2, *E* and *F*). In each case, most of the ligand was present in intracellular vesicles following 30 min at 37°C. Relative to the CT18 deletion mutant, little ligand remained on the cell surface as indicated by immunofluorescence using nonpermeabilized cells (not shown).

To examine more quantitatively the effects of the various

Table I. Accumulation of Gold-labeled IgG complexes in Coated Pits and Coated Vesicles in Transfected CHO Cells

Receptor	Gold in coated pits and vesicles (relative)	Gold in coated pits and vesicles (percent)	Total gold counted (number)	Average number of receptors per cell
B2	100	7.7	851	0.7×10^5
B1	0	0.0	2254	4.0×10^5
B2 CT31	40	3.1	2605	2.1×10^5
B2 CT18	7	0.53	2447	1.2×10^5
B2 tail-minus	7	0.52	2290	2.6×10^5
B2 A26	86	6.6	1014	1.1×10^5
B2 A43	78	6.0	1095	7.4×10^5
B2 A26&43	73	5.6	1176	3.5×10^5
B2/B1-R	6	0.44	2250	6.7×10^5
B2/B1-W	92	7.1	1084	1.6×10^5

Transfected CHO cells grown in 22-mm tissue culture wells were incubated with 20 μg/ml gold-conjugated IgG-complexes for 2 h at 0°C. Cells were washed with cold PBS-glucose, rapidly warmed to 37°C for 1.5 min, immediately fixed in glutaraldehyde, and processed for EM as described in Materials and Methods. Random micrographs were taken at 10,000× and the quantitation of gold particles on the plasma membrane, in coated pits and coated vesicles was performed using prints enlarged to a final magnification of 25,000×. In each case, based on previous determinations, surface membrane corresponding to a total contour length of at least 0.75 mm was analyzed. FcRII-B2 A26 and -B2/B1-W were quantified directly from the electron microscope screen. The data for FcRII-B1, B2 and B2 tail-minus have in part been published earlier (Miettinen et al., 1989) and are in part new (direct quantitation from the electron microscope screen). The number (or area) of coated pits per cell did not exhibit significant differences between cell lines. The number of receptors per cell was measured by determining the binding of ^{125}I-2.4G2 Fab at 0°C (Miettinen et al., 1989).

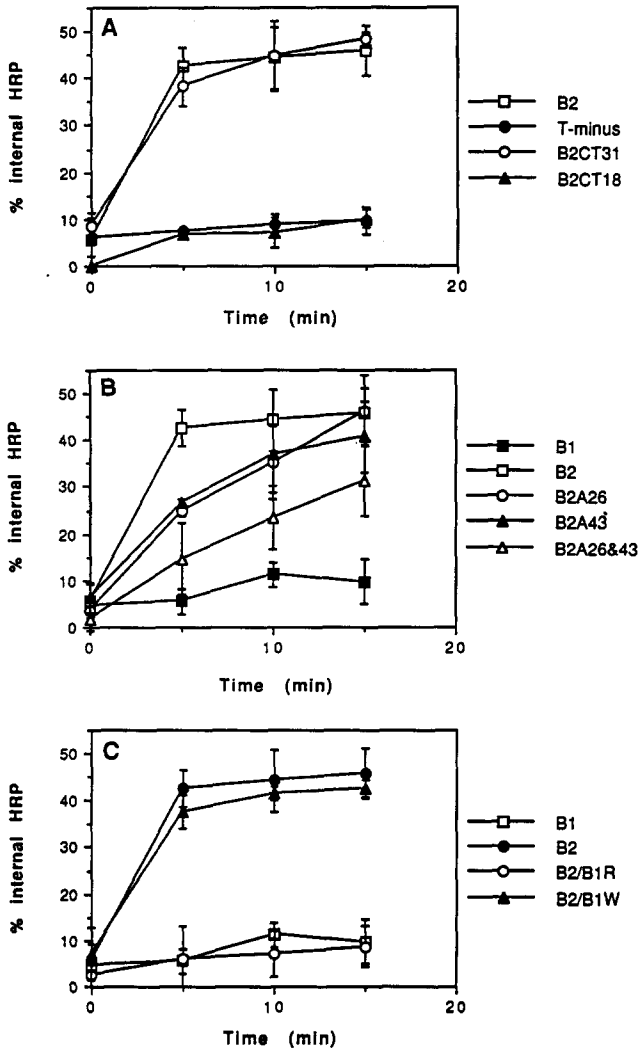

Figure 3. Internalization of IgG-complexes in CHO cells expressing wild type and mutant FcRII. CHO cells expressing wild type FcRII or various receptor mutants were grown in 22-mm wells and allowed to bind immune complexes consisting of HRP/anti-HRP IgG on ice. After washing, the cells were then rapidly warmed to 37°C.

mutations on endocytosis, we next determined the kinetics of internalization using a biochemical assay. For this purpose, stable FcRII-expressing cell lines were generated by transfecting CHO cells with an amplifiable expression vector (pFRSV) and selected in increasing concentrations of methotrexate (Miettinen et al., 1989). Cells were then sorted in a fluorescence-activated cell sorter so that 3–5% of the highest expressing cells were collected, yielding cell lines expressing 0.7×10^5 to 7.4×10^5 receptors/cell, similar to FcRII expression in macrophages (Table I). The cell lines were screened for FcRII-mediated endocytosis by immunofluorescence and found to yield patterns similar to those obtained for the transiently expressing COS-1 cells (Fig. 2).

To measure endocytosis, IgG immune complexes, formed using HRP and affinity-purified anti-HRP, were bound on ice, unbound ligand removed by washing, and the cultures warmed to 37°C. After various time points, surface-bound and intracellular HRP was determined by measuring HRP activity in the absence or presence of Triton X-100, respectively (Drake et al., 1989). Internalized ligand was taken as the difference between these two values. Binding and uptake were judged to be specific since both signals were reduced by >95% by including the anti-FcRII mAb 2.4G2 IgG, an antibody that effectively blocks ligand binding to FcRII on intact cells (Mellman and Unkeless, 1980; Mellman and Plutner, 1984). As shown in Fig. 3 *A*, cells transfected with

At the indicated time points, the cells were returned to ice and endocytosis determined by measuring the amount of total vs. cell surface HRP-IgG as described in Materials and Methods. Internalized HRP was calculated by subtracting the amount of surface HRP from the amount of total HRP. The amount of HRP activity released into the medium accounted for <5% of the initially-bound activity; 100% of the initial HRP activity was recovered at each time point. All determinations were performed in duplicate and the data represent the means of 2-3 different experiments (±SD). (*A*) FcRII-B2 and the B2 deletion mutants tail-minus, CT31, and CT18. (*B*) FcRII-B1 and -B2, and the B2 tyrosine to alanine mutants A26, A43, and A26& 43. (*C*) FcRII-B1 and -B2, and the B1 insert mutants B2/B1R and B2/B1W.

either wild type FcRII-B2 or the CT31 deletion mutant mediated rapid internalization of the IgG-complexes, with uptake being complete within 5 min after warming. In contrast, cells expressing the CT18 or tail-minus deletion mutants exhibited little if any ligand. Thus, using the HRP-complex assay for endocytosis, the differences between B2 and tail-minus transfectants were much more pronounced than observed using the previously described protease-release assay which was shown to remove surface-bound IgG-complexes only inefficiently (Miettinen et al., 1989). The HRP-complex uptake data also yielded kinetics for FcRII-B2 (and CT31) uptake that were very similar to IgG-complex endocytosis by B2-expressing macrophages (Mellman and Plutner, 1984).

As indicated by the immunofluorescence experiments, cells expressing the A26 or A43 tyrosine mutants were also capable of rapid internalization of bound ligand, although the rate of uptake was slowed slightly (but significantly) relative to FcRII-B2 (Fig. 3 *B*). Interestingly, this rate was further slowed in the case of the double tyrosine mutant (A26 and 43) to <50% of the B2 wild type. Nevertheless, even the double mutant-mediated internalization of the IgG-complexes at a rate many-fold greater than the tail-minus deletion mutant or FcRII-B1 (Fig. 3 *B*). The results of the biochemical experiments further support the suggestion that the conserved region (residues 18–31) of the FcRII-B2 cytoplasmic domain at least in part contains a determinant that is required for rapid endocytosis. Moreover, they indicate that the tyrosine residue within this region itself is at best only partially required. While elimination of both cytoplasmic domain tyrosines reduced the internalization rate significantly, it was clear that the effect of this mutation was far less than complete.

FcRII Tyrosine Residues Are Not Required for Coated Pit Localization

We have previously found that the endocytosis of IgG-complexes by FcRII-transfected CHO cells can proceed in part by a coated pit–independent mechanism resembling phagocytosis. Therefore, to determine whether the immunofluorescence and biochemical assays for endocytosis reflected the abilities of the different FcRII mutants to accumulate at coated pits, we next measured coated pit localization directly using quantitative EM. Monolayers were incubated at 0°C with gold-conjugated IgG-complexes, washed, warmed to 37°C for 1.5 min, and then fixed in glutaraldehyde. For each transfectant, the following parameters were analyzed from a minimum of 0.75 mm total cell surface contour length: the total number of gold particles, the number of gold particles in coated pits and coated vesicles, and the number of coated pits and coated vesicles. The percent of total gold found in coated pits was determined and the efficiency of coated pit localization expressed relative to cells expressing the FcRII-B2 wild type. Binding was judged to be specific since cell-associated gold was reduced >90% by simultaneous incubation with the anti-FcRII antibody 2.4G2 (Miettinen et al., 1989). The number (or area) of coated pits per cell was not significantly different among the various cell lines. The results are summarized in Table I.

As found previously, CHO cells expressing FcRII-B2 accumulated 7.7% of the gold IgG-complexes in coated pits after 1.5 min at 37°C. This extent of coated pit localization was similar to that found for FcRII in macrophages (Ukkonen et al., 1986; unpublished results) as well as for transferrin receptor in fibroblasts (Miller et al., 1991). The various FcRII-B2 tyrosine mutants A26, A43, and A26 and 43, were similar to the wild type receptor with 6.6%, 6.0% and 5.6% of the total gold-IgG complex in coated pits, respectively. Thus, relative to cells transfected with FcRII-B2, these cell lines were 73–86% as efficient at coated pit localization. These results are in agreement with the immunofluorescence and endocytosis data suggesting that the loss of one or both tyrosine residues had only a minor or incomplete effect on internalization of ligand.

Cells transfected with the FcRII-B2 deletion mutants, however, exhibited decreased abilities to accumulate gold-labeled ligand in coated pits. CT31, the mutant lacking the last 16 amino acids (including the tyrosine at position 43), exhibited a significant but incomplete defect. As shown in Table I, this mutant was 40% as efficient at coated pit localization as was full length FcRII-B2 (3.1% of the total gold in coated pits/vesicles). Thus, receptors devoid of the COOH-terminal third of the cytoplasmic tail were still able to accumulate at coated pits with significant, but reduced, efficiency. This was in contrast to the endocytosis data which indicated that CT31 mediated IgG-complex uptake as efficiently as the B2 wild type.

More dramatic, and consistent with the biochemical data, was the phenotype exhibited by cells expressing the CT18 mutant, whose cytoplasmic tail was deleted through the region of homology between the human and mouse receptors. The CT18 transfectants were as inefficient at coated pit localization as cells transfected with the tail-minus mutant. In both cell lines, only 0.5% of the gold was found at coated regions, corresponding to 7% of the efficiency of the FcRII-B2 transfectants (Table I). Thus, deletion of residues 18–31 reduced the ability of the receptor to localize at coated pits as much as a complete truncation of cytoplasmic tail. While these results indicate that the conserved region is important for coated pit localization, they do not exclude the possibility that other regions of the cytoplasmic tail also play a role, particularly since the CT31 mutant had a partial effect on coated pit localization as did the elimination of both tyrosine residues.

FcRII-B1 Insert Actively Prevents Coated Pit Localization

We previously found that the lymphocyte isoform of FcRII (FcRII-B1) does not localize in coated pits and is poorly internalized (Miettinen et al., 1989). FcRII-B1 is identical to FcRII-B2 except for an insertion of additional 47 amino acids that lengthens its tail to 94 residues (Fig. 1). Having found that the insert occurs in a region of the cytoplasmic tail that was not clearly required for coated pit localization, it became of interest to further characterize how the FcRII-B1 insert abolished endocytosis.

The insert might prevent accumulation at coated pits by an active mechanism or by somehow masking or disrupting the coated pit localization determinant. Given that FcRII-B1 is much less efficient at coated pit localization than the tail-minus mutant (Table I) (Miettinen et al., 1989), it seemed likely that the insert worked via an active mechanism. If so, the insert might exert an inhibitory effect regardless of its po-

sition in the cytoplasmic tail. To test this possibility, we constructed a receptor containing the entire FcRII-B2 cytoplasmic domain with the B1 insert added to its COOH terminus. A unique MluI restriction site was first created at the end of the B2 coding sequence by site-directed mutagenesis. This mutation changed the two last amino acids (histidine and isoleucine) to threonine and arginine (Fig. 1). By immunofluorescence in COS-1 cells, this change did not affect endocytosis of the receptor (not shown). The B1 insert was then synthesized by polymerase chain reaction and cloned at the MluI site either in the right (B2/B1-R) or in the wrong (B2/B1-W) orientations (Fig. 1). We next established stable CHO cell lines expressing the receptors and determined their ability to internalize and degrade IgG-complexes.

Addition of the B1 insert to the COOH terminus of FcRII-B2 (B2/B1-R) significantly slowed the rate of ligand uptake relative to FcRII-B2. As shown in Fig. 3 *C*, cells transfected with the B2/B1-R were as inefficient at ligand uptake as FcRII-B1. The inhibition was not due simply to the presence of additional amino acid residues at the FcRII-B2 COOH terminus. This was demonstrated by the fact that cells transfected with a receptor containing the insert in the wrong orientation (B2/B1-W), a construct that yielded 19 random amino acids at its COOH terminus, had an internalization rate that was indistinguishable from cells transfected with the FcRII-B2 wild type (Fig. 3 *C*).

To determine whether the different behavior of these receptors was due to differences in their abilities to localize in coated pits, we next directly assayed coated pit localization by EM. As expected from the endocytosis data, cells expressing the B2/B1-W construct mediated coated pit localization at an efficiency comparable to the FcRII-B2 wild type (Fig. 4 *A* and Table I). In contrast, the B2/B1-R mutant exhibited a marked reduction in coated pit localization (Fig. 4 *B*). Only 0.44% of the total gold was found in coated pits after 1.5 min at 37°C, corresponding to a 17–18-fold decrease in efficiency relative to wild type (Table I). Thus, the FcRII-B1 insert prevented receptor localization in coated pits irrespective of its position in the cytoplasmic tail, suggesting that the insert contains information that actively prevents the receptor from accumulating in coated pits.

FcRII-B1 Insert Causes Receptor Alignment with Actin Filaments and Entry into a Detergent-insoluble Fraction

The FcRII-B1 insert might prevent coated pit localization in one of two ways, either by selectively excluding the receptor from coated pits or by retaining the receptor at uncoated regions of the plasma membrane. Conceivably, such a "retention signal" might work by tethering FcRII-B1 to the cytoskeleton. We had previously observed that a fraction of

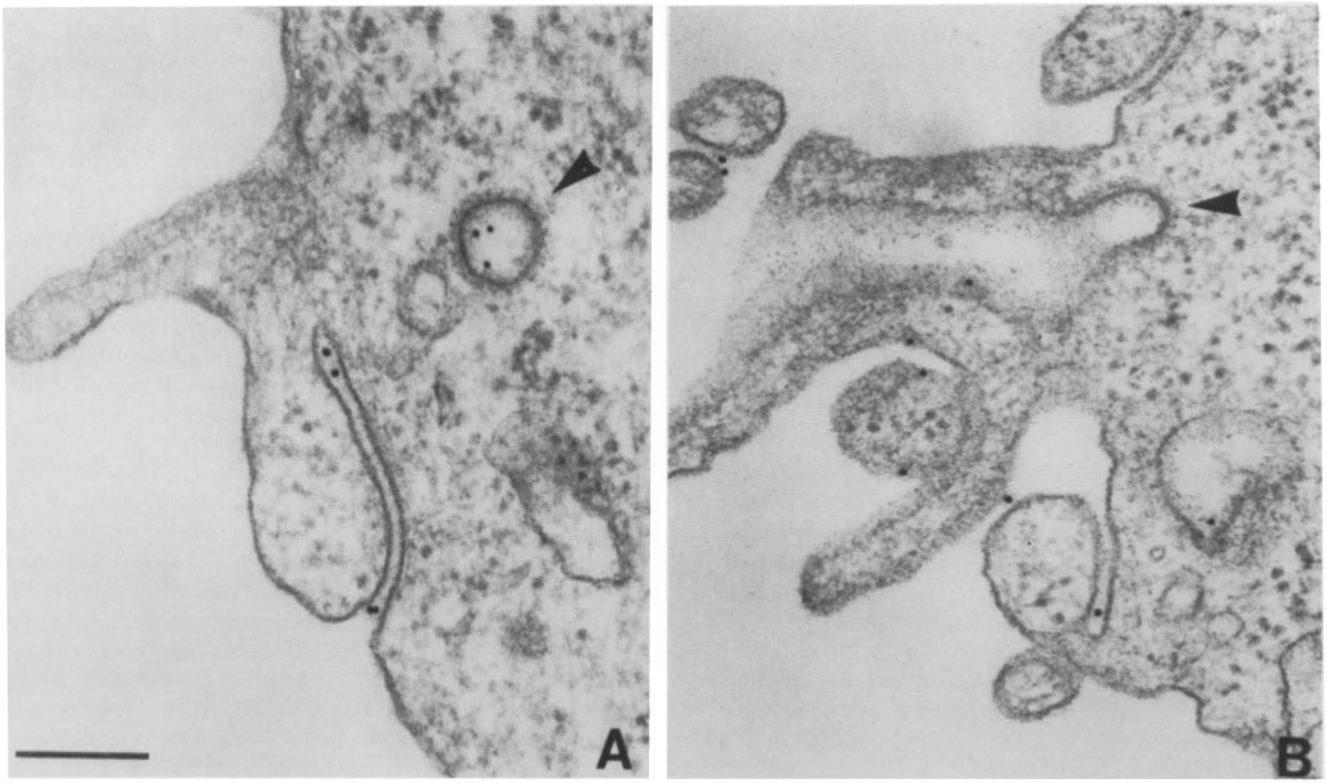

Figure 4. Distribution of gold-labeled IgG-complexes in transfected CHO cells. CHO cells expressing either FcRII-B2/B1-R or B2/B1-W, chimeric receptors containing the FcRII-B1 insertion at their COOH-terminals in the right (*R*) or wrong (*W*) orientations, were grown in 22-mm wells and incubated with 20 μg/ml gold-conjugated IgG complexes for 2 h at 0°C. After washing with PBS-glucose to remove unbound ligand, the tissue culture plates were dipped for 1.5 min into complete prewarmed (37°C) medium, immediately fixed in glutaraldehyde, and processed for thin section EM. (*A*) Cells expressing the B2/B1-W chimera had numerous gold particles is coated pits and coated vesicles (7.1% of the total cell-associated gold, summarized in Table I). (*B*) Gold particles were only rarely found in cells expressing the B2/B1-R receptor construct (0.44% of the total, see Table I). Bar, 0.2 μm.

ligand bound to FcRII-B1 on the cell surface formed linear arrays after incubation at 37°C (Miettinen et al., 1989), suggesting a possible cytoskeleton association. This staining pattern was never observed in cells expressing FcRII-B2 or the endocytosis-defective CT18 or tail-minus mutants. Thus, the linear arrays did not simply reflect poor internalization, but were specific for FcRII-B1.

We next determined whether cells expressing the FcRII-B2 constructs with the COOH-terminal B1 insert in the right (B2/B1-R) or wrong (B2/B1-W) orientations also exhibited the linear arrays and whether the arrays corresponded to any cytoskeletal element. Transfected CHO cells were incubated with prebound IgG-complexes for 1 h at 37°C, fixed, and processed for immunofluorescence. As shown in Fig. 5, cells expressing either FcRII-B1 or the B2/B1-R mutant (*A* and *C*) clearly showed that a fraction of the receptor-ligand complexes was found in linear arrays. In contrast, cells transfected with B2/B1-W (*E*) exhibited a pattern of intracellular vesicular staining similar to the FcRII-B2 transfectants (Fig. 2 *A*). To determine whether the arrays localized with any cytoskeletal elements, the cells were costained with antibodies against fodrin and tubulin, and with rhodamine-conjugated phalloidin to visualize F-actin. While the antifodrin and antitubulin staining did not show any colocalization with the linear arrays, the actin filament staining indeed colocalized (Fig. 5 *B*, *D*, and *F*), suggesting a direct or indirect interaction between the receptor and the actin microfilament network.

To further characterize the interaction, we examined the time course for the formation of the linear arrays. CHO cells expressing FcRII-B1 were incubated with IgG complexes for 2 h at 0°C, warmed to 37°C for 5, 10, and 20 min, and prepared for immunofluorescence. Although the linear arrays were not visible at 5 min, they began to appear after 10 min at 37°C and to colocalize with actin (Fig. 6). Thus, the putative interaction between FcRII-B1 and cytoskeleton was both time and temperature dependent.

If the FcRII-B1 insert specified an interaction between the receptor and the cytoskeleton, it might also be expected to cause receptors containing the insert to become resistant to detergent extraction. To determine if this was the case, cells were labeled with [^{35}S]cysteine and then placed on ice for 2 h or incubated for 30 min at 37°C in the presence or absence of ligand (IgG-complexes or polyclonal anti-FcRII antibody). The cells were washed with PBS and extracted on ice for 10 min with 0.5% Triton X-100 (Fey et al., 1984). Monolayers were scraped with a rubber policeperson and the detergent-insoluble fraction was pelleted by centrifugation. The pellet was then solubilized with SDS, and FcR immunoprecipitated.

As shown in Fig. 7 *A*, when CHO cells expressing FcRII-B1 were incubated in the absence of ligand at 0°C, labeled receptor was not precipitated from the Triton X-100 insoluble fraction (lanes *1* and *2*); similar results were obtained for cells incubated at 37°C without ligand (not shown). If IgG-complexes were added to the cells at 0°C, a small fraction of the receptor could be precipitated from the insoluble pool (lanes *3* and *4*). However, incubation with IgG-complexes or rabbit anti-FcR antibody at 37°C resulted in the recovery of 25–35% of the total FcRII-B1 from the detergent insoluble fraction (lanes *5–8*, *arrowhead*). Similar results were obtained when the cells were first incubated 2 h on ice with the ligand and then warmed up to 37°C for 30 min (not shown). Thus, in agreement with the conditions required for the formation of linear receptor arrays, appearance of FcRII-B1 in the detergent-insoluble fraction was both ligand and temperature dependent. It also occurred rapidly, with kinetics that were similar to the appearance of the linear arrays. As shown in Fig. 7 *B* and *C*, Triton-insoluble receptor was barely detectable after 5 min at 37°C, but reached a plateau after 10 min.

Importantly, detergent-insoluble receptor was not detected in cells expressing FcRII-B2 even after prolonged incubations under any conditions (0°C or 37°C, in the presence or absence of either ligand) (Fig. 7 *A*, lanes *9–12*). However, a significant amount (~30%) the FcRII-B2 construct containing the FcRII-B1 insert at its COOH terminus (B2/B1-R) was recovered from the Triton-insoluble fraction after incubation with ligand at 37°C (lanes *13* and *14*). The FcRII-B2 construct containing the insert in the wrong orientation remained Triton-soluble (lanes *15* and *16*). Thus, the presence of the FcRII-B1 insert—at the proximal or distal regions of the cytoplasmic tail—resulted in the ligand and temperature-dependent association of the receptor with a detergent-insoluble fraction expected to contain the cytoskeleton.

Discussion

Analysis of naturally occurring variations in the FcRII cytoplasmic tail has begun to reveal how alternative mRNA splicing can determine the function of plasma membrane proteins (Amigorena, S., C. Bonnerot, D. Choquet, G. Raposo, J.-G. Guillet, C. Sautes, W. H. Fridman, J. Drake, W. Hunziker, P. Webster, and I. Mellman, manuscript submitted for publication; Hunziker et al., 1990; Joiner et al., 1990; Miettinen et al., 1989). Evidence presented in this paper suggests that although FcRII isoforms with distinct abilities for endocytosis both contain an intact coated pit localization domain, its function is specifically inactivated by the sequence insertion in FcRII-B1. Moreover, the coated pit localization domain itself appeared to be somewhat different from that typically associated with endocytic receptors.

The three other receptors (LDL-, mannose-6-phosphate-, and transferrin receptors) whose coated pit localization domains have been studied in greatest detail were each found to contain a cytoplasmic tail tyrosine whose presence was strongly required for rapid endocytosis and/or accumulation at coated pits (Alvarez et al., 1990; Davis et al., 1987; Gironès et al., 1991; Jing et al., 1990; Lobel et al., 1989; McGraw and Maxfield, 1990). In each case, alteration of a single tyrosine residue reduced the rate or efficiency of endocytosis by 5–20-fold. For LDL and transferrin receptors, this decrease was also found to reflect a similar decrease in receptor accumulation at coated pits. There is ample evidence that these tyrosines form part of an as yet incompletely understood sequence motif whose secondary structure is thought to represent a recognition site for adaptor or assembly proteins found in clathrin-coated pits (Pearse and Robinson, 1990).

The FcRII-B2 cytoplasmic tail contains two tyrosines at positions 26 and 43, and no other aromatic amino acids. Y26 was the best candidate for a residue critical for coated pit localization for two reasons. First, it was found within the only region of the cytoplasmic tail that is highly conserved between murine and human FcRII (Stuart et al., 1989). Sec-

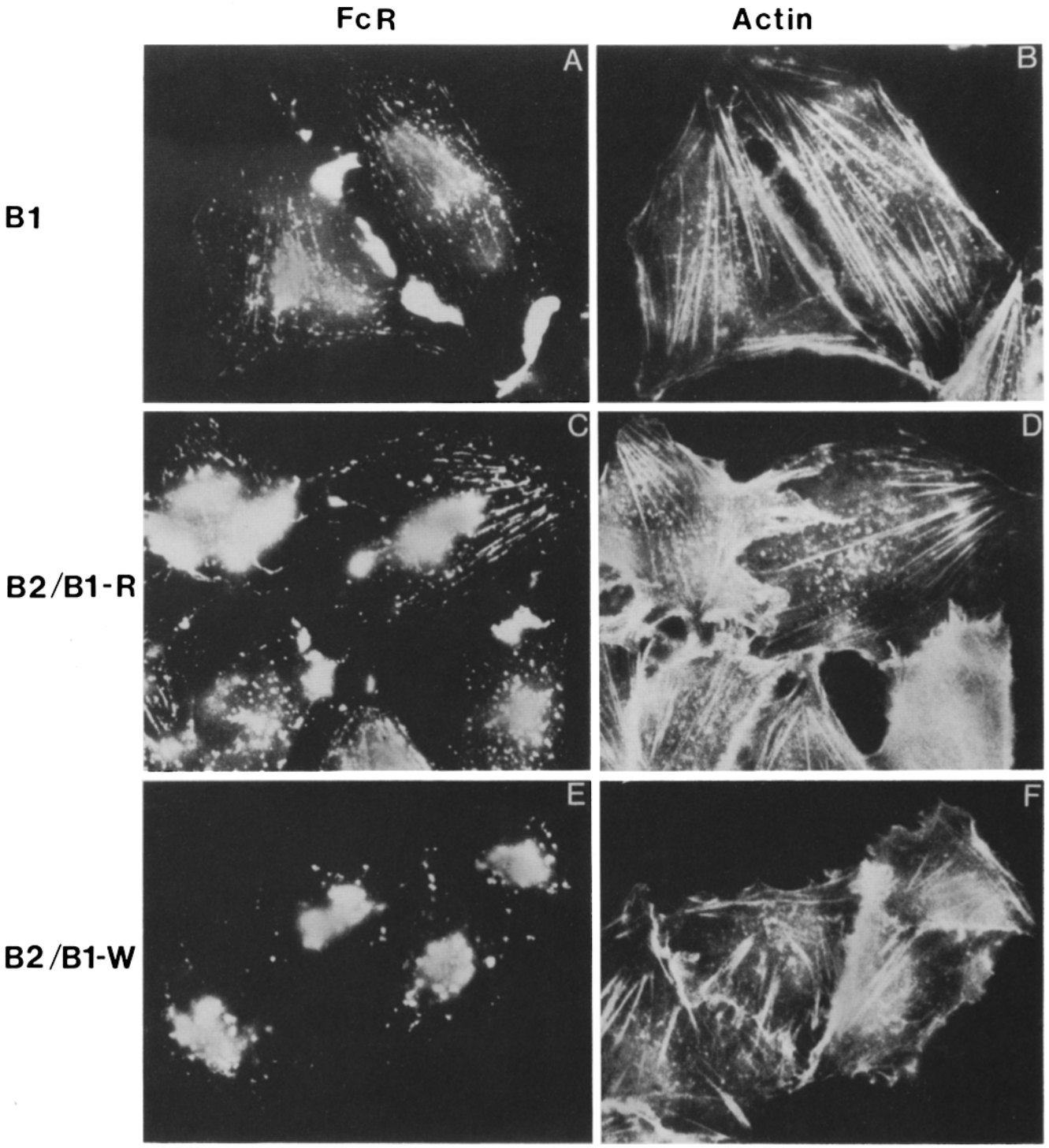

Figure 5. Fc receptors containing the FcRII-B1 insert form linear arrays that colocalize with F-actin. Stably transfected CHO cells were incubated 2 h at 0°C with 20 μg/ml IgG-complexes, washed, and then warmed in complete medium to 37°C. After 30 min, the cells were fixed with paraformaldehyde-lysine-periodate and permeabilized with saponin. Receptor-ligand complexes were visualized using $F(ab')_2$ fluorescein-conjugated goat anti-rabbit IgG and actin filaments using rhodamine-conjugated phalloidin. (*A*) FcRII-B1, IgG-receptor complexes; (*B*) FcRII-B1, actin filaments; (*C*) FcII-B2/B1-R, IgG-receptor complexes; (*D*) FcRII-B2/B1-R, actin filaments; (*E*) FcRII-B2/B1-W, IgG-receptor complexes; (*F*) FcRII-B2/B1-W, actin filaments.

ond, the sequences surrounding Y26 are at least superficially similar to sequences found in other coated pit receptors. One recent secondary structure prediction suggests that the residues on the amino-terminal side of the tyrosine tends to be a random coil that may form a salt bridge with polar residues just downstream of the tyrosine (Ktistakis et al., 1990). The NPXY sequence motif defined as important for the LDL receptor coated pit localization domain (Chen et

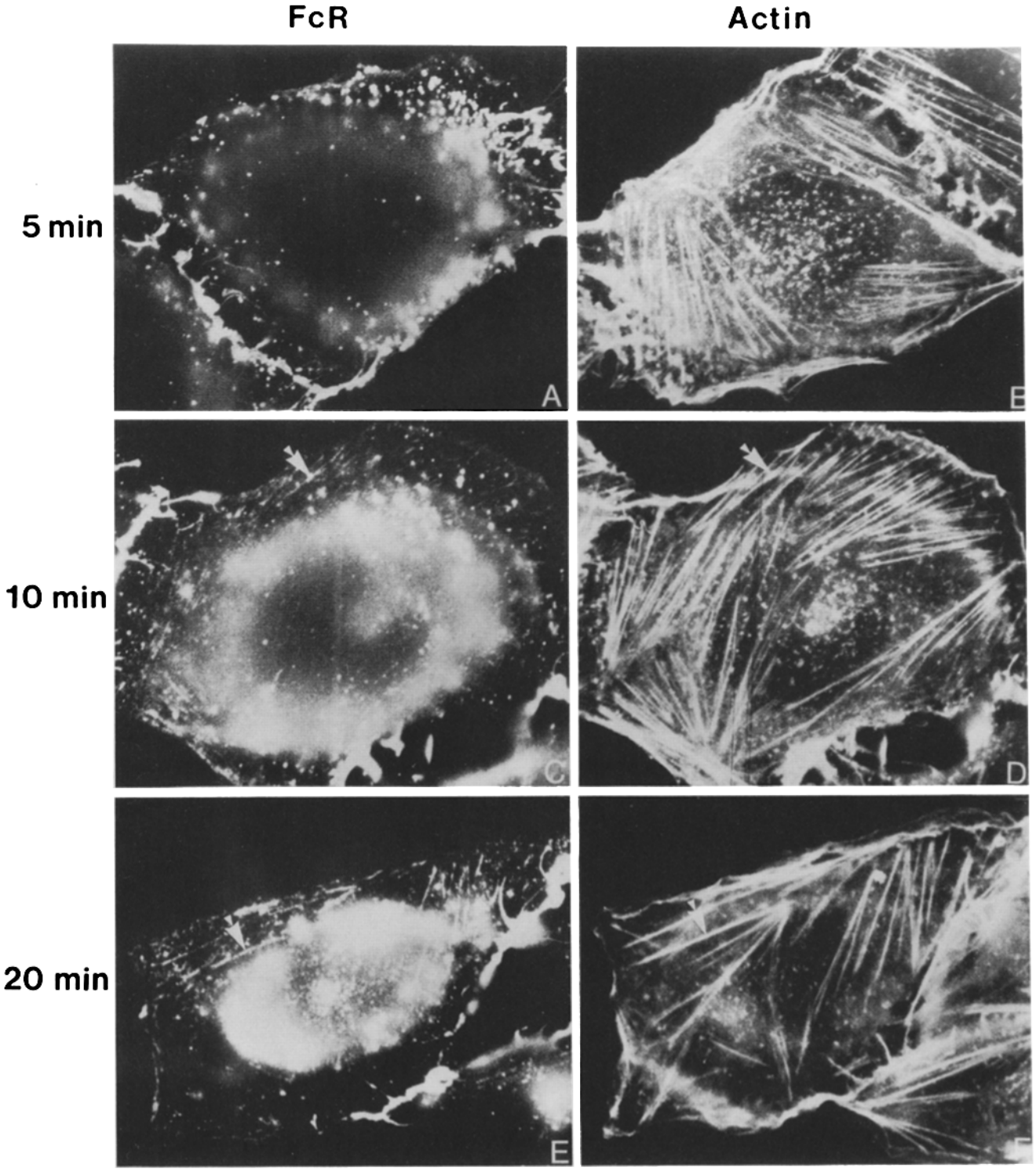

Figure 6. Time course for formation of linear arrays in CHO cells expressing FcRII-B1. Cells were incubated for 2 h at 0°C in medium containing 20 μg/ml IgG complexes, washed, and warmed to 37°C for 5, 10, or 20 min before fixation. Fixed cells were permeabilized with saponin and incubated with fluorescein-conjugated goat anti-rabbit IgG to visualize receptor-bound ligand (*A*, *C*, and *E*) and with rhodamine-conjugated phalloidin to visualize actin filaments (*B*, *D*, and *F*). (*A* and *B*) 5 min; (*C* and *D*) 10 min; (*E* and *F*) 20 min.

al., 1990) is included in this prediction. While not as strongly predicted, the sequence around Y26 (Fig. 1) possessed the expected features (Ktistakis et al., 1990).

We were thus surprised to find that changing either Y26 or Y43 to alanines only slightly decreased the rate of IgG-complex internalization and did not appreciably decrease the efficiency of coated pit localization. Changing both tyrosines simultaneously, interestingly, did reduce endocytosis by ~50%. This decrease did not reflect a major reduction in the frequency of FcRII localization at coated pits, which re-

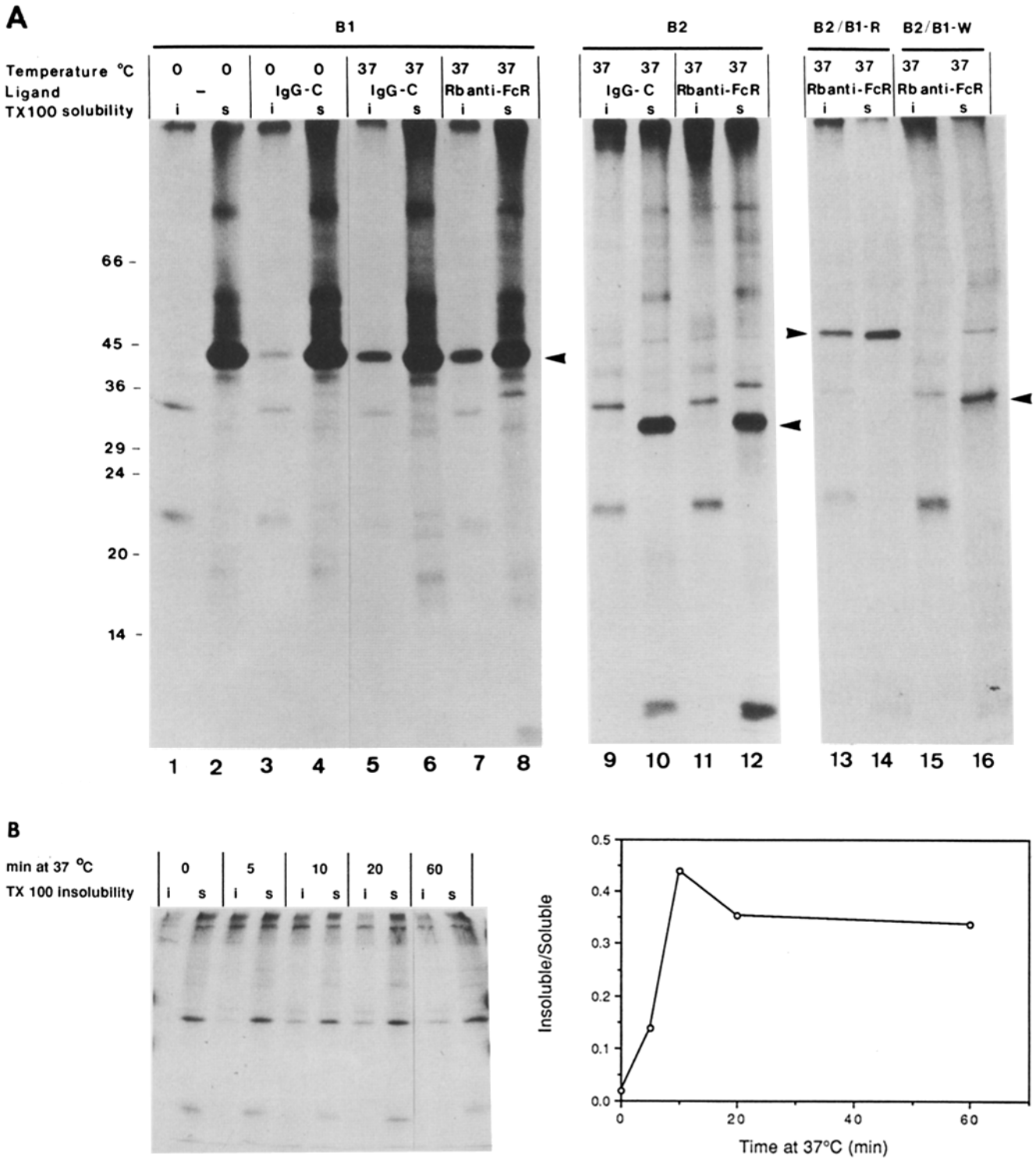

Figure 7. Immunoprecipitation of FcRII from insoluble and soluble fractions of Triton X-100 extracted CHO transfectants. Cells were labeled with ^{35}S-cysteine, washed, chased for 1 h at 37°C, and incubated for an additional 30 min at 0°C or 37°C with or without 20 μg/ml IgG-complexes or a monospecific polyclonal rabbit anti-FcRII serum (1:100 final dilution). The cultures were washed with cold PBS-glucose and extracted with 0.5% Triton X-100 in 10 mM K-Pipes, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, and 1 mM PMSF (Fey et al., 1984). Labeled FcRII was then immunoprecipitated from both the Triton X-100 soluble and insoluble fractions (after solubilization in SDS) using a rabbit anti-FcRII serum. The precipitated samples were treated with N-glycanase and then analyzed by SDS-PAGE and autoradiography. Autoradiographs were quantified using a Kodak Visage 2000 image processor. Since FcRII normally runs as a diffuse glycoprotein band after SDS-PAGE, the glycosidase treatment was used to facilitate quantitation. The migration of molecular weight markers (10^{-3}) is shown at left. (*A*) Triton X-100 solubility of FcRII and FcRII (*arrows*) in the presence or absence of ligand at 0°C and 37°C. *IgG-C*, IgG complexes; Rb anti-FcR, rabbit anti-FcRII anti-serum; *i*, insoluble; *s*, soluble. (lanes *1–8*) cells transfected with FcRII-B1; (lanes *9–12*) FcRII-B2; (lanes *13* and *14*) FcRII-B2/B1-R; (lanes *15* and *16*) FcRII-B2/B1-W. (*B*) Time course of appearance of Triton X-100 insolubility of FcRII-B1 after exposure to IgG-complexes at 37°C. (*C*) Quantitation of the autoradiogram shown in *B*.

mained at 80% of control. Moreover, endocytosis by the Y26 and 43 FcRII mutant was considerably less affected than observed for mutations of even multiple tyrosines (or aromatic amino acids) in other receptors such as the transferrin, polymeric immunoglobulin, and cation-dependent mannose-6-phosphate receptors (Breitfeld et al., 1990; Gironès et al., 1991; Johnson et al., 1990). This was true even for the asialoglycoprotein receptor in which deletion of the single cytoplasmic tail tyrosine only partially eliminated coated pit localization and endocytosis, but nevertheless reduced both by approximately threefold (Fuhrer et al., 1991). Although the synergistic effect of altering Y26 and Y43 together is consistent with there being at least two regions of the FcRII cytoplasmic domain which may be involved in coated pit localization, as suggested for the cation-dependent mannose-6-phosphate receptor, it would appear that tyrosine residues may have a relatively reduced importance in FcRII relative to most other receptors. However, the lack of an absolute dependence on the presence of a tyrosine residue appears to be a more general phenomenon that had originally been thought.

Analysis of the deletion mutants suggested that the cytoplasmic tail did appear to contain specific regions that were required for coated pit localization. Perhaps the most important was a 13 residue segment containing Y26 and corresponding to the region of homology between the human and mouse receptors. Truncation of the cytoplasmic tail up to the homologous region (CT31) did not decrease the rate of IgG-complex internalization. It did cause a partial but significant decrease in coated pit localization (40% relative to FcRII-B2 wild type). The reason for the disparity between the biochemical and morphological assays is unclear, but may reflect the difficulties and variability inherent to quantitative EM: it should also be emphasized that quantitative correlations between biochemistry and EM has been attempted for few other receptors. The possible role of variation in the morphological assay is emphasized by the fact that deletions (CT18) removing the entire homologous region reduced both endocytosis and coated pit accumulation to levels identical to that of a mutant lacking the entire cytoplasmic tail. Thus, the region between amino acids 19–31 may play a more important role in specifying coated pit localization than the first 18 or the COOH-terminal 16 amino acids. However, deletion mutations causing loss of function cannot be used alone to identify this domain definitively.

Taken together, these results suggest that FcRII-B2 contains a coated pit localization domain that does not require a tyrosine or other aromatic amino acid for activity. The existence of a second, tyrosine-independent signal may reflect the fact that receptors such as FcRII-B2 are not constitutively concentrated at coated pits. For example, up to 70% of all LDL receptors can be found in coated pits which account for only 2–3% of the cell surface (Anderson et al., 1982). On the other hand, when determined by thin section EM, only 6% of total transferrin receptors or asialoglycoprotein receptors in fibroblasts are found at coated pits (Fuhrer et al., 1991; Miller et al., 1991). FcRII are more evenly distributed and may accumulate at coated pits only after binding multivalent IgG-complexes (Miettinen et al., 1989; Ukkonen et al., 1986). When FcRII distribution is visualized using monovalent antireceptor Fab–gold conjugates on glutaraldehyde-fixed cells, no more than 1% of the receptors are found in coated pits (unpublished results). Thus, the FcRII-B2 coated pit localization domain may be subject to regulation, its activity being enhanced by ligand binding. This may also hold for other receptors, e.g., polypeptide growth factors, whose internalization may also be regulated by ligand binding. The coated pit localization domains of growth factor receptors have not yet been precisely defined, thus it is not clear what type of coated pit localization determinant is used. Interestingly, EGF receptor has three consensus NPXY sequences in the distal segment of its >500 residue cytoplasmic tail (Ullrich, 1984). Deletion of this segment, however, has no effect on the rate of EGF endocytosis (Chen et al., 1989).

Irrespective of the number or types of coated pit localization domains that are ultimately identified, it can be assumed that they must all directly or indirectly be recognized by one or more components of coated pits. Conceivably, all compete for the same pit components. In this case, each domain should have structural features in common, regardless of the role of tyrosine, possibly explaining the predicted relationship of the FcRII-B2 sequence to tyrosine-containing signals (Ktistakis et al., 1990). An appealing possibility is that the common element of this structure may be a tight turn, based on an analysis of a crystallographic data base (Collawn et al., 1990).

A Signal That Blocks Endocytosis and Coated Pit Localization

It is clear that the function of the FcRII-B2 coated pit localization domain is abrogated by the FcRII-B1 insertion (Miettinen et al., 1989). Our results now show this effect to be more a function of the insert itself than of its location in the FcRII tail. In murine and human FcRII, alternative splicing places the insert at an intron–exon junction corresponding to residue 6 of the murine cytoplasmic tail (Qiu et al., 1990). Since this site is 12 residues away from a presumptive coated pit localization domain (i.e., residues 19–31), its effect is not due to physical disruption. This is consistent with the recent finding that only sequences corresponding to the amino-terminal half of the insertion are capable of blocking endocytosis when inserted at position 6 in the FcRII-B2 cytoplasmic domain (Matter, K., and I. Mellman, unpublished observations). Since the COOH-terminal half of the insertion alone is without effect, it is apparent that specific, as opposed to random, sequences must be present at position 6 in order to mediate the block in coated pit localization. The insertion does not have any obvious sequence homology to other known proteins, however.

Moreover, transplanting the insert to the COOH terminus of the FcRII-B2 tail (B2/B1-R) resulted in an almost complete inhibition of coated pit localization. The B2/B1-R construct was somewhat less efficient than FcRII-B1, however, suggesting that the insert's position in the tail may have some role in determining its effect. Nevertheless, these results show that the insertion contains information that is necessary and sufficient to prevent FcRII localization at coated pits.

It was also clear that the FcRII-B1 insertion must do more than simply inactivate the FcRII-B2 coated pit localization domain. Removal of the domain, as in the tail-minus or CT18 mutants, reduced the frequency of coated pit localization to 7% of control, presumably reflecting random distribution of receptor. In the FcRII-B1 transfectants, however, virtually no receptor was observed associated with coated pits out of over 2,250 gold particles counted. Thus, FcRII-B1 must be either

selectively excluded from entering coated pits or actively retained in uncoated regions of the plasma membrane. Given that the insert also appeared to cause the receptor to associate with the cytoskeleton—or at least to align with actin filament and to enter a detergent-insoluble fraction—its effect may be thought of as a "retention signal" that prevents the receptor from entering coated regions. This possibility was supported by the fact that the appearance of FcRII-B1 in the detergent insoluble fraction apparently occurred almost as fast ($t_{1/2}$ ~5 min) as FcRII-B2–mediated endocytosis. Although ligand-induced association with the cytoskeleton may occur rapidly enough to interfere with the ligand-induced accumulation of the receptor at coated pits, we cannot yet conclude that this is the mechanism by which the insert blocks internalization. Instead, the insert might allow the receptor to interact with another protein which is already excluded from coated pits, with cytoskeletal attachment of this complex occurring as a secondary phenomenon. If this possibility is true, then a region of the FcRII-B1 cytoplasmic domain other than the insert itself may actually be responsible for detergent insolubility. This possibility is consistent with preliminary results showing that cytoskeletal attachment requires not just the insert but sequences distal to the insert (Matter, K., and I. Mellman, unpublished data).

Several receptors and nonreceptor plasma membrane proteins are already known to associate with the cytoskeleton. In nucleated cells, one of the best characterized examples is the Na^+,K^+-ATPase which binds to fodrin in conjunction with ankyrin and uvomorulin in kidney epithelial cells (McNeill et al., 1990; Morrow et al., 1989; Nelson and Veshnok, 1987). As in the case of FcRII-B1, the detergent-insoluble fraction is incomplete (20–50%) depending on the degree of cell confluence or cell polarity. The fact that <100% of a membrane protein can be recovered as insoluble suggests that association with the cytoskeleton is a dynamic event, with individual receptors continuously forming and breaking the associations that lead to detergent insolubility. In support of this possibility are preliminary experiments in which living cells expressing FcRII-B1 were viewed by differential interference contrast microscopy. Individual ligand-conjugated gold particles were found to exist in two states on the plasma membrane, the first corresponding to restricted linear movements and the second to apparently random diffusion in all directions (unpublished data). While more direct evidence will be required to establish that FcRII-B1 actually binds directly or indirectly to one or more elements of the cytoskeleton, it is interesting that the fraction of the receptor that is recovered in the detergent-insoluble fraction can be increased significantly (greater than twofold) by performing the extraction in the presence of phalloidin, an agent that stabilizes actin filaments (Matter, K., and I. Mellman, unpublished data).

Our results show that alternative splicing can regulate a receptor's ability to internalize ligand via clathrin-coated pits apparently by introducing a sequence that binds the receptor to the cytoskeleton. While we have used transfected fibroblasts, it is very likely that the insert performs an analogous function in B-cells where FcRII-B1 is normally expressed. Most B-cells and B-cell lines express high levels of FcRII-B1 which plays an important role in regulating surface immunoglobulin-induced B-cell activation (Bijsterbosch and Klaus, 1985; Phillips, 1984). These cells are incapable of internalizing and degrading receptor-bound IgG-complexes or of presenting IgG-conjugated antigen to T-cells (Amigorena et al., 1991). Significantly, transfection of FcRII-B2 into receptor-negative B-cell mutants completely corrects both of these "defects." Since FcRII-B1 typically cocaps with cross-linked surface immunoglobulins, and since cap formation is accompanied by a local reorganization of cytoplasmic actin and *ras* (Graziadei et al., 1990), it is possible that the receptor's ability to interact with the cytoskeleton plays a role in the formation or function of the cap itself.

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