Isolation and Characterization of Chinese Hamster Ovary Cell Variants Deficient in the Expression of Fibronectin Receptor

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Abstract. Chinese hamster ovary cell populations were enriched for cells displaying low surface expression of the 140-kD integrin fibronectin receptor (FnR) by means of fluorescence-activated cell sorting using monoclonal anti-FnR antibodies. Selected cells were cloned by limiting dilution, and the resulting clones were screened for low cell surface FnR expression by ELISA. Two multiply sorted populations gave rise to variant clones possessing ~20 or 2% FnR expression, respectively, compared with wild-type cells. Growth rates of the "20%" and "2%" clones on serum-coated plastic dishes were similar to that of wild-type cells. Variant cells expressing 20% FnR could attach and spread on substrata coated with purified fibronectin, although somewhat more slowly than wild-type cells, while cells expressing 2% FnR could not attach or spread. Cells from all variant clones attached normally to vitronectin substrata, but some of the 2% clones displayed altered morphology on this type of substratum. Motility assays in blind well chambers showed a correlation of movement with level of expression of FnR. The number of cells migrating in response to fibronectin was greatly reduced compared with wild-type cells for the 20% FnR variant clones, while variant clones with 2% FnR showed virtually no migratory activity.

Surface labeling with ^{125}I and immunoaffinity purification of FnR showed reduced levels of intact FnR on the plasma membranes of variants with 20% FnR, while none was detected in variants expressing 2% FnR. Nevertheless, β subunits were detected on the surfaces of all variant clones. Immunoblots of cell lysates from wild-type cells and from both types of variant clones showed substantial amounts of FnR β chain as well as enhanced amounts of a pre-β moiety in the variants. α chain was markedly reduced in the 20% variants and essentially absent in the 2% variants, indicating that failure to assemble intact FnR in these variants was due to deficiencies of α chain production. Dot blots of total mRNA from a representative clone expressing 20% FnR showed reduced levels of material hybridizing to an 0.97-kb hamster FnR α chain cDNA probe as compared with wild type, while mRNA from a representative clone expressing 2% FnR had no detectable hybridizable RNA; this seems to agree well with the results obtained by immunoblotting. Thus, the defect in the variant clones seems to be due to reduced levels of α chain mRNA leading to a deficit of mature FnR and consequent alterations in cell adhesion and motility on fibronectin substrata.

THE cell surface receptor for fibronectin (FnR)[1] plays a major role in mediating adhesive interactions between cells and the extracellular matrix (11, 31, 45). This intrinsic membrane glycoprotein of ~140 kD is a member of the integrin superfamily of cell adhesion receptors. Integrins share a number of common features; for example, they are composed of noncovalently linked heterodimers with large extracellular domains. In mammalian cells, the integrin receptor superfamily is divided into three subfamilies on the basis of three different smaller (β) subunits that are shared by a number of larger unique (α) subunits. (Additional β subunits have recently been described [13, 32.]) The β_1 subfamily includes the FnR (in human cells designated as $\alpha^5\beta_1$) as well as at least five other known $\alpha^x\beta_1$ proteins, including receptors for collagen and laminin (Ln) (28, 36, 50, 54–56). The second subfamily defined by the β_2 subunit is mainly involved in leukocyte cell–cell interaction and, as such, participates in immune and inflammatory responses (33–35). The third, or β_3, subfamily includes the receptors for vitronectin (VnR) (44, 52) and thrombospondin (37) as well as platelet glycoprotein IIb/IIIa, a receptor for multiple ligands (fibrinogen, von Willebrand's factor, vitronectin [Vn], and fibronectin [Fn]) (22, 23, 42). Recently, great progress has been made in the molecular cloning and structural analysis of members of the integrin superfamily. In both α and β chains, a large extracellular amino-terminal domain is followed by a single membrane-spanning helix and a relatively

1. *Abbreviations used in this paper*: CHO, Chinese hamster ovary; FACS, fluorescence-activated cell sorting; Fn, fibronectin; FnR, fibronectin receptor; Ln, laminin; Vn, vitronectin; VnR, vitronectin receptor.

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small (~20–50 amino acids) internal carboxy-terminal domain; the β subunit contains a cysteine-rich domain that is extensively S-S bridged, while α subunits have several cation-binding domains (3, 4, 16, 29, 33, 43, 53).

The biosynthesis and assembly of integrins has also been examined recently; it seems that assembly of the $\alpha\beta$ heterodimer is required for ligand binding activity and that association precedes maturation of the complex in the Golgi apparatus and expression of the functional integrin on the cell surface (1, 12, 27). The binding of ligands by many, but not all, integrins involves recognition in the ligand molecule of a tripeptide sequence, Arg-Gly-Asp (RGD), found in numerous extracellular matrix proteins; however, regions flanking the RGD sequence, as well as more distant sites, may determine the affinity and specificity of interactions between protein ligands and integrin receptors (31, 40, 45, 57). Recently, the putative binding sites for the RGD sequence have been identified in the β chains of gp IIb/IIIa and the VnR by use of photoactivatable RGD-containing peptides (18, 49). It has also become clear recently that some aspects of integrin function in cells are regulated by protein kinase C and by cyclic nucleotide–dependent kinases (17). Thus, the biochemical basis of integrin function is beginning to be defined.

Cellular interactions mediated by integrins, including those involving the FnR, are vital to many significant physiological and pathological processes. This would include embryonic cell migration (19, 20), cell and tissue differentiation (39), tumor cell metastasis and invasion (24, 30), cytoskeleton–plasma membrane associations (51), and modification of the morphology and behavior of transformed cells in culture (11, 12). Studies of the role of the FnR in particular cellular or developmental processes have usually relied on interference with normal ligand–receptor binding by means of proteolytic fragments of fibronectin, synthetic RGD peptides, or antibodies to Fn and/or FnR (6, 24, 30, 38, 51, 57). Such results are equivocal in that these moieties may or may not have similar effects upon binding to the FnR as does the authentic ligand; further, the magnitude and duration of these effects may be quite variable in cell culture or during in vivo situations. We therefore sought an unambiguous way to delineate the role of the FnR in various cellular processes and, for that purpose, have isolated a series of stable FnR-deficient variants from the Chinese hamster ovary (CHO) cell line, using monoclonal antibodies to FnR as tools for enrichment and selection. A similar strategy could readily be applied to any other integrin receptor for which antibodies are available. Specifically, these variants should allow us, first, to determine the relative importance of the FnR, compared with other integrins and with nonintegrin-mediated mechanisms, in determining cellular adhesion, morphology, and motility on various extracellular matrix substrata. Second, the intracellular signals generated upon ligation of FnR are unknown; having a true negative control may allow for their discovery. Third, elucidation of receptor function at the molecular level should be facilitated by using these variants as transfection hosts for intact or modified integrin cDNAs. Fourth, study of FnR-deficient cells may provide important insights into the regulation of integrin expression.

In this study, we have selected a series of CHO clones which are either partially or virtually completely deficient in the cell surface expression of FnR. These clones were selected by using monoclonal antibodies directed against intact FnR or against the β subunit of the receptor. Our studies include evaluation of growth characteristics, cell adhesion, cell motility, surface expression, and total cellular expression of FnR, as well as assessment of messenger RNA levels for this integrin.

Materials and Methods

Cell Culture

CHO cells were grown and maintained as previously described (17, 26) either as monolayers in α-MEM (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS (Hazelton Research Products, Lenexa, KS) and 1% antibiotic-antimycotic mixture (Gibco Laboratories) or in suspension culture where the FCS was reduced to 5%. Monolayers were routinely passaged with trypsin-EDTA (Gibco Laboratories), and cell number was determined with an ElectroZone celloscope (Particle Data, Inc., Elmhurst, IL).

Variant Cell Enrichment

Subconfluent cell monolayers were harvested with 0.02% EDTA in PBS at 4°C, or log-phase suspension-grown cells were washed three times with cold PBS. All subsequent operations were performed at 4°C in α-MEM containing 1% BSA, maintaining sterility. Cells were stained by indirect immunofluorescence, using anti-FnR mouse monoclonal antibodies PB1 (specific for the intact Chinese hamster FnR) or 7E2 (specific for Chinese hamster β_1 chain) as primary antibody (7, 9) and FITC-conjugated rabbit anti-mouse IgG (Cappell-Organon Technika, Malvern, PA) as a secondary reagent. Antibody staining reactions were virtually complete after 15 min and were followed by extensive washing in α-MEM, 1% BSA. Background staining was assessed by omitting primary antibody. Cells were separated by fluorescence-activated cell sorting (FACS) on an Epics IV flow cytometer (Coulter Electronics Inc., Hialeah, FL) equipped with a 488-nm argon laser and were gated for viability (forward angle light scatter) and low fluorescein fluorescence. Cells selected for low fluorescence were initially plated directly into 96-well plates at three cells per well and regrown to confluence. Cultures with low anti-FnR binding were then batch-sorted two to four additional times, until the mean fluorescence of the population of sorted cells was ~40% or less of the intensity of the unsorted CHO cell population. The final batch-sorted populations were allowed to recover for 2–3 d and then cloned by limiting dilution at 0.5 cells/well and screened by ELISA.

Screening and Selection

Progeny of individual clones were screened for low FnR expression in intact cells by ELISA. Cells were plated in microtiter plates precoated with polylysine (1 mg/ml; 50 μl/well) and gelatin (0.1%; 100 μl/well) and were screened using PB1 or 7E2 mAb as primary antibody followed by sheep anti-mouse β-galactosidase conjugate and enzyme substrate according to the manufacturer's instructions (Bethesda Research Laboratories, Gaithersburg, MD). Plates were read at 405 nm on a microplate autoreader (Bio-Tek Instruments, Inc., Winooski, VT). Selected clones with low FnR expression were rescreened over a period of 1 mo to be certain that receptor expression was stable. These clones were also evaluated in terms of their expression of a cell surface antigen (6C10) unrelated to integrins, using a monoclonal antibody to this molecule (6), and in terms of expression of VnR, a member of the β_3 subfamily of integrins, using a polyclonal anti-VnR antibody (Telios, La Jolla, CA).

Adhesion Assay

Bovine plasma Fn was prepared as previously described (48). Vn was prepared from human plasma as described by Ruoslahti et al., (46) except that fast performance liquid chromatography (ion exchange) was performed on a Mono Q column (Pharmacia Fine Chemicals, Piscataway, NJ) with a 20 mM Tris starting buffer, pH 7.6, in the absence of urea. Ln was obtained from Collaborative Research (Bedford, MA). Fn, Vn, and Ln substrata were prepared by allowing varying concentrations of these proteins to adsorb to wells of tissue culture–grade 48-well plates for 2 h at 37°C. The plates were then washed in PBS, blocked with 3% BSA in PBS for 1 h at 37°C, and finally washed four times with α-MEM containing 1% BSA and 20 mM Hepes, pH 7.3 (adhesion buffer). Cells were metabolically labeled

during overnight growth in suspension culture with ^{35}S-Trans label (2-5 μCi/ml, 1,000 Ci/mM; ICN Radiochemicals, Irvine, CA), washed three times, and resuspended in adhesion buffer to a concentration of 1-5 $\times$ 10^5 cells/ml. Attachment was allowed to proceed in a 37°C incubator for varying times, at the end of which unattached cells were removed by washing the substrata with adhesion buffer (17). Attached cells were lysed with 2% SDS, and the lysate was transferred to scintillation vials for counting. All assays were performed in triplicate, and the results are expressed as either a percentage of the total number of cells or as a percentage of the positive control.

Cell Motility Assays

Cell motility in the presence of Fn was measured essentially as described by Albini et al. (2) in Boyden blind cell chambers (Nuclepore, Pleasanton, CA) fitted with 13-mm polyvinylpyrrolidone-free polycarbonate filters (8 μm pore size). The lower chambers were filled with 3T3 cell-conditioned medium (2) supplemented with 30 μg/ml Fn. Suspension-cultured, washed CHO cells (3-5 $\times$ 10^5) in α-MEM containing 0.1% BSA were added to the upper chambers to initiate the experiment. After a 9-h incubation at 37°C, the medium was removed from the upper chambers and any cells remaining on the upper surfaces of the filters were removed by scraping with cotton-tipped applicators. Cells on the undersides were fixed by flooding with Carnoy's fixative and stained with hematoxylin and eosin. Filters were then mounted on slides, and cells in random microscopic fields were counted. Under the described conditions, ~200 wild-type cells were counted on the filter's lower chamber side per microscope field. All experiments were performed in triplicate.

Immunoaffinity Resins

Antibody-coupled Affigel 10 resins were prepared according to Brown and Juliano (7) as modified by Sczekan, M. M., and R. L. Juliano (manuscript submitted for publication). Antibodies used were 7E2 mAb (anti-CHO β_1 chain), PB1 mAb (anti-CHO intact FnR), and anti-gp140, a goat anti-FnR polyclonal antibody (7, 9).

Surface Labeling and Analysis

Suspension-grown CHO cells, washed three times in cold PBS, were radioiodinated with Na^{125}I (200 μCi/10^7 cells; ICN Radiochemicals) using Iodo-Gen according to the manufacturer's instructions (Pierce Chemical Co., Rockford, IL). Labeled cells were incubated with lysis buffer (0.5 ml/2 $\times$ 10^7 cells) containing 10 mM Tris, pH 7.4, 150 mM NaCl, 3 mM iodoacetamide, and 100 mM *n*-octyl-β-D-glucopyranoside on ice for 1 h. Lysates were clarified by centrifugation at 12,000 *g* for 10 min. The supernatant fractions were then gently mixed overnight at 4°C with immunoaffinity resin that had been equilibrated in lysis buffer. After washing the pelleted resin three times with lysis buffer, adsorbed protein was eluted from the resin by boiling for 5 min in SDS-sample buffer without β-mercaptoethanol followed by centrifugation to pellet the resin. Eluates representing equivalent cell numbers were electrophoresed on 7.5% polyacrylamide gels as described by Laemmli (37). Gels were dried and autoradiographed with XAR film (Eastman Kodak Co., Rochester, NY).

Immunoblotting

Lysates of unlabeled cells were prepared as described for surface labeling. FnR protein was partially purified using wheat germ agglutinin coupled to agarose (Vector Laboratories, Burlingame, CA) and with an immunoaffinity resin. Protein was eluted and electrophoresed as described above, transferred to nitrocellulose, blotted with anti-gp140 polyclonal antibody followed by ^{125}I-protein A, and autoradiographed as described by Brown and Juliano (8) as modified by Sczekan, M. M., and R. L. Juliano (manuscript submitted for publication).

Preparation of cDNA Probes

A cDNA representing part of the sequence of hamster FnR α chain was obtained as follows. CHO cell poly A$^+$ RNA (14) was used to prepare cDNAs using avian myeloblastosis virus reverse transcriptase and oligo dT priming (41). The cDNAs were used to construct a λgt11 expression library (Stratagene, La Jolla, CA) that contained ~1.6 $\times$ 10^6 recombinants. The gt11 vectors were plaqued on *Escherichia coli* Y1090, and lysis and expression were induced. The plaques were then probed with our goat anti-FnR affinity-purified antibody (anti-gp140) followed by rabbit anti-goat IgG and ^{125}I-protein A. Positive plaques were recloned until all plaques tested positive. Antibodies bound to fusion proteins from positive plaques, when subsequently eluted, bound specifically to the α but not β chain of authentic FnR (data not shown). Thus, the positive clones likely represented FnR α cDNA. Positive gt11 clones were restricted with Eco RI, and the inserts were sized by agarose gel electrophoresis; selected inserts ranged from 0.93 to 2.25 kb. Inserts were subcloned into the pGEM3 plasmid vector (Promega Biotec, Madison, WI) followed by transformation of *E. coli* AG1 with the vector (25). Subcloning success was determined by restricting out the inserts from purified plasmid and comparing insert size with that of the original gt11 vectors by agarose gel electrophoresis. Using the T7 and SP6 promoters of the pGEM3 vector, we have partially sequenced an 0.97-kb insert using the dideoxy technique (47). Sequencing 1-200 bases into each site and use of homology search programs (International Biotechnologies, Inc., New Haven, CT) indicates substantial homology to the published sequence for human FnR α chain (3). Our insert seems to lie in the middle of the coding region approximately from bases 880 to 1,810. Conversion to amino acid sequence indicates stretches of almost perfect homology to human FnR α. Partial amino acid sequence comparison of hamster 0.97-kb FnR α clone with human FnR α is as follows. The hamster and human FnR clone SP6 sites are EDFVAGVP and EDFVAGVP, respectively; the hamster and human FnR clone T7 sites are GALEDCREMKIYL-RNESE and GAREDCREMKIYLRNESE, respectively. A full sequence of the hamster FnR α chain is being completed (Hussein, S., and R. L. Juliano, manuscript in preparation). Radioactive (^{35}S) cDNA probes were prepared from the hamster α chain 0.97-kb insert from the Bluescribe plasmid (as a negative control) and from a β_1 cDNA (as a positive control) by random priming (21) using a protocol from the Promega Biotec technical manual. A human β_1 chain cDNA clone was kindly provided by Dr. L. Fitzgerald (Cor Therapeutics, South San Francisco, CA).

Dot Blots

Total RNAs from wild-type and FnR-deficient CHO cell variants were prepared (14). Hybridizations were done in dot-blot format using a slight modification of the procedure described by Perbal (41), including 40 mM sodium phosphate in the hybridization solutions.

Results

Variant Selection and Growth Characteristics

Early attempts at directly selecting variants expressing low levels of FnR by a single flow cytometric run resulted in cells with unstable levels of receptor expression, presumably either because normal cells overgrew deficient variants or because populations initially identified as low expressors were under some unknown transient stress. Therefore, the strategy of iterative FACS was used. Cells were first sorted by FACS with an autoclone attachment at three cells per well and allowed to regrow to confluence. Gated cells represented <0.1% of the population. Of the 10^7 cells sorted, only 3 of the 253 wells plated contained low expressors as determined by ELISA. Two of the three resulting populations were then expanded and batch-sorted several times; the third population was frozen away for future use. The first population then required four batch sorts followed by regrowth periods before cloning by limiting dilution resulted in clones with stable, low expression of FnR, while the second population required two batch sorts. Examples of fluorescence intensity histograms of these populations made at the beginning of the final sort are shown in Fig. 1 (the first and second populations are shown in Fig. 1, *A* and *B*, respectively, as peaks 2). Compared with wild-type CHO cells, the population means of log-integrated green fluorescence for low FnR-expressing populations are shifted leftward. The first population displays a mean fluorescence intensity of ~34% of wild type, while that of the second population is ~43% of wild type; further, in both populations, there are a substantial number

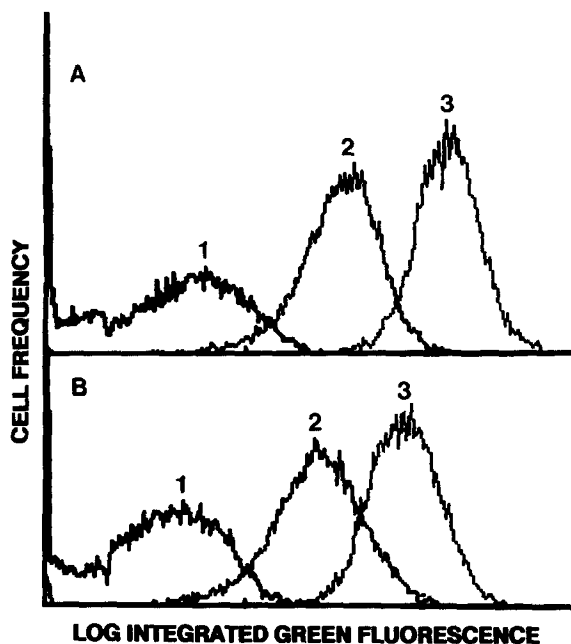

Figure 1. FACS histograms of variant populations of CHO cells before final sorting. Equivalent numbers of wild-type and variant cells were washed with cold PBS, then treated with anti-FnR monoclonal antibodies followed by staining with fluorescein-conjugated rabbit anti-mouse IgG, and subjected to fluorescent analysis before sorting. Each peak represents 10^4 cells. (*A*) First population or wild-type cells treated with 7E2 monoclonal as primary antibody. (*B*) Second population or wild-type cells treated with PB1 monoclonal antibody. (Peaks *1-3*) nonspecific staining (no primary antibody), variant cell population, and CHO wild-type cells, respectively.

of cells that overlap the profile of the negative control (unstained) population. Having obtained populations enriched in low expressing cells, it was then possible to efficiently clone and screen enough cells to find the rare FnR-deficient variants we sought. Using this approach it is not possible to determine the frequency of the FnR-deficient variants in the wild-type population since several rounds of enrichment occurred before final screening and selection.

Based on the data from ELISAs using PB1 mAb, which binds only to the intact heterodimer, the clones arising from the first population all express ~20% as much intact receptor as wild-type cells, while clones arising from the second population all express only ~2% of wild-type levels (Table I). (Since variations of 2% are near the level of experimental error of the ELISA assay, our designation of clones as expressing "2%," of FnR simply means that very low levels of expression were observed.) There is no way of knowing if this is simply happenstance, if there is some bias in the individual enrichment processes, or if all the clones isolated from each population represent the progeny of a single variant cell in each population. It should also be noted that the levels of expression of the FnR mAb epitopes PB1 and 7E2 are not equivalent in the low expressing cells. There is much more 7E2 epitope than PB1 epitope, especially in the clones arising from the second population. For the sake of convenience we will henceforth refer to clones originating from the first enriched population as "20%" clones and those originating from the second population as "2%" clones, realizing that

*Table I. ELISA of CHO Clones**

Clone	Primary antibody		
	PB1	7E2	6C10
1-23	15 ± 8	24 ± 14	97 ± 11
14-16	24 ± 2	34 ± 8	92 ± 4
21-4	18 ± 8	24 ± 8	103 ± 7
B2	2 ± 1	20 ± 7	87 ± 17
D1	1 ± 2	24 ± 5	91 ± 11
9	1 ± 2	20 ± 4	92 ± 17
23	1 ± 2	23	95 ± 13
25	0.4 ± 0.8	20 ± 2	96 ± 1

* Results are expressed as the mean ± SD of at least four experiments using PB1 and 6C10 as primary antibodies and two experiments using 7E2 as primary antibody done over a period of at least 1 mo. Each experiment consisted of at least triplicate samples of each clone tested. Data are expressed as the percentage of the positive control, CHO wild-type cells.

there are in fact subtle differences among individual clones within each group.

To ascertain that the low level of binding of anti-FnR antibodies did not represent a general defect in cell surface glycoprotein display, ELISAs were done using an irrelevant mAb (6C10) against a nonintegrin CHO cell surface glycoprotein (6). All clones deficient in FnR showed a normal level of 6C10 antigen present on their surfaces as compared with wild-type cells (Table I). Preliminary observations using a commercial anti-VnR antibody suggest that FnR-deficient variants display normal amounts of VnR on their surfaces (data not shown).

Growth curves of clones from both populations were compared with that of wild-type cells. Clones from the first population (1-23, 14-16, and 21-4) expressing ~20% of wild-type levels of FnR and clones B2 and 25 from the second population behaved identically to wild-type cells with average doubling times of ~14 h. Three of the clones from the second population (expressing ~2% of wild-type levels of FnR) grew somewhat more slowly. Clone D1 had a doubling time of 18 h, while clones 9 and 23 had doubling times of 20 h. There seems to be no correlation of growth rates with the level of expression of receptor since some clones with very low FnR expression have equivalent doubling times to wild-type cells: for example, 15 h for clone B2 and 14 h for clone 25.

Adhesion to Substrata

Maximum adhesion of wild-type cells occurred on surfaces coated with 1 μg/ml Fn (Fig. 2). Clones expressing levels of receptor at 20% of wild type (1-23, 14-16, and 21-4) displayed similar adhesive efficiencies as wild-type cells on surfaces coated with various amounts of Fn. Almost no cells from clones displaying ≤2% FnR (clones B2, D1, 9, 23, and 25) adhered to substrata coated with increasing amounts of Fn within the 90 min of the experiment (see below). To assess whether FnR expression at a level of 20% of wild type places other limitations on the variants' abilities to adhere to Fn-coated substrata, we examined how rapidly these clones could adhere to a limiting concentration of Fn on the substratum. At a concentration of 0.2 μg/ml Fn, clones expressing FnR at 20% of wild type adhered more slowly than wild-type cells (Fig. 3), but, at the end of the 90-min incubation period, the fraction of adherent cells approached that of wild

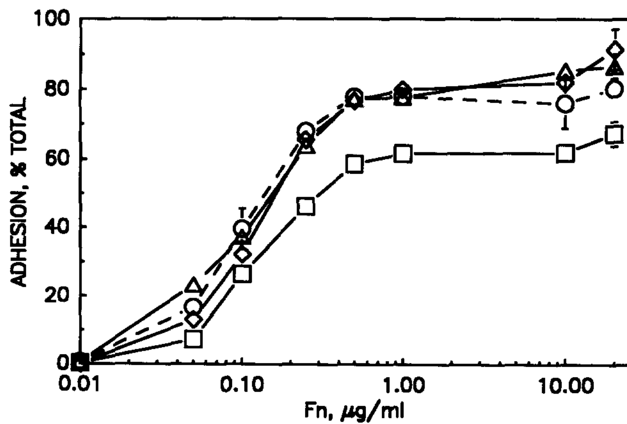

Figure 2. Efficiency of adhesion of CHO cell variants to Fn-coated substrata. Tissue culture plates were coated with various concentrations of Fn for 2 h at 37°C in PBS and then blocked with BSA as in Materials and Methods. Cells of wild-type or 20% clones metabolically labeled overnight with ^{35}S-Trans label were washed in PBS and resuspended at 5 × 10^5 cells/ml and allowed to adhere for 1.5 h at 37°C. The adherent cells were recovered and analyzed for radioactivity as described, and the number of adherent cells was calculated. (○) Wild-type cells; (△) clone 1-23; (□) clone 14-16; (◇) clone 21-4.

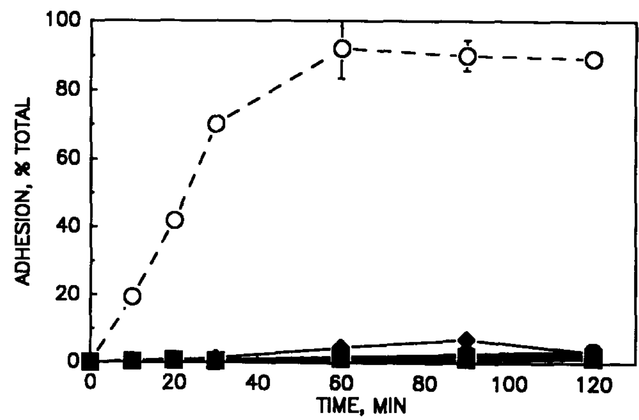

Figure 4. Kinetics of adhesion of CHO variants to a nonlimiting concentration of Fn. Tissue culture plates were coated with 1 µg/ml of Fn and then blocked with BSA. CHO wild-type and 2% variant cells were prelabeled with ^{35}S as described in Materials and Methods, washed, and resuspended at 10^5 cells/ml in α-MEM containing 1% BSA and 20 mM Hepes, pH 7.3, and allowed to adhere at 37°C for different time intervals. The attached cells were recovered and analyzed for radioactivity as described, and the number of adherent cells was calculated. (○) Wild-type cells; (●) clone B2; (▲) clone D1; (▼) clone 9; (■) clone 23; (◆) clone 25.

type. These results suggest that only when receptor expression falls below ~20% of wild type, is the cell's ability to form stable adhesions to Fn-coated substrata markedly impaired. In other words, there seem to be numerous "spare receptors" for Fn-mediated adhesion in wild-type cells, as we have previously noted (8). By contrast, clones displaying ≤2% FnR do not adhere to substrata coated with nonlimiting concentrations of Fn (1 µg/ml) even after 2 h (Fig. 4).

Similar experiments were repeated on Vn-coated substrata. In all cases the variant cells behaved essentially the same as wild type: adhesion to increasing amounts of Vn was maximum at ~5 µg/ml (data not shown). All variants displayed kinetics of adhesion similar to wild-type cells over a 2-h time course on Vn substrata (Fig. 5). With Vn at a limiting concentration (0.5 µg/ml), clones expressing 20% FnR compared with wild-type cells were at least as efficient as wild-type cells in forming stable adhesions (Fig. 5 *A*). At a nonlimiting concentration of Vn (5 µg/ml), clones expressing 2% of wild-type levels of FnR were also identical in behavior to wild-type cells (Fig. 5 *B*); this should be contrasted with the parallel experiment on Fn substrata (Fig. 4). The 20% clones were also tested on Ln substrata and were found to attach as efficiently as wild-type cells (data not shown). These results indicate that the variant populations have an adhesion defect only for Fn substrata, while other integrin-mediated adhesive mechanisms remain functional.

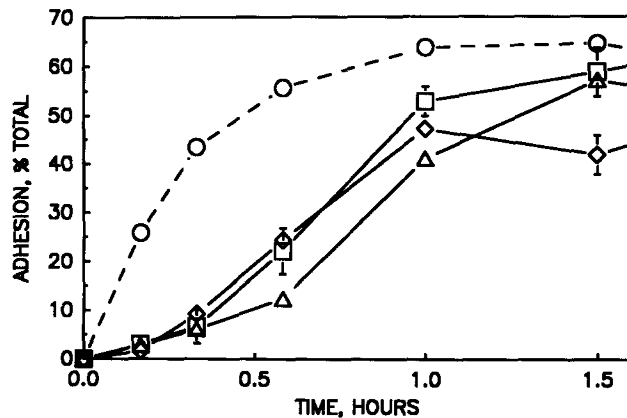

Figure 3. Kinetics of the adhesion of variants to a limiting concentration of Fn. Tissue culture plates were coated with 0.2 µg/ml of Fn and then blocked with BSA. CHO wild-type cells and 20% variants were metabolically labeled with ^{35}S as described in Materials and Methods, washed, resuspended to a concentration of 10^5 cells/ml in α-MEM containing 1% BSA and 20 mM Hepes, pH 7.3, and allowed to adhere at 37°C for different time intervals. The attached cells were recovered and analyzed for radioactivity as described, and the number of adherent cells was calculated. (○) Wild-type cells; (△) clone 1-23; (□) clone 14-16; (◇) clone 21-4.

Phase-contrast photomicrographs reveal differences in ability to spread on Fn- or Vn-coated substrata among different clones. A representative clone from each population is shown in Fig. 6. A variant expressing 20% FnR (clone 1-23) spread nearly as well as wild-type cells on Fn, but the cell margins were irregular. Those few cells from a variant expressing 2% FnR (clone 25) that adhered on a high concentration of Fn were neither spread nor flattened. On 5 µg/ml Vn, clones expressing 20% FnR were indistinguishable in morphology from wild-type cells. Clones expressing 2% FnR demonstrated some variation in their response on Vn: clones B2 and 25 flatten and also spread to a slight extent, but clones D1, 9, and 23 neither flatten nor spread although they adhere quite well. Thus, the ability of cells to adhere to a substratum (see Fig. 5) is clearly distinct from the ability of those cells to spread on the same substratum. It is not known whether the variation in the morphologies of 2% variants on Vn is due to subtle differences in VnR abundance or to clone-to-clone differences in cytoskeletal or metabolic activities involved in cell spreading. The failure of the variants expressing 2% FnR to spread on Vn substrata suggests that FnR and VnR cooper-

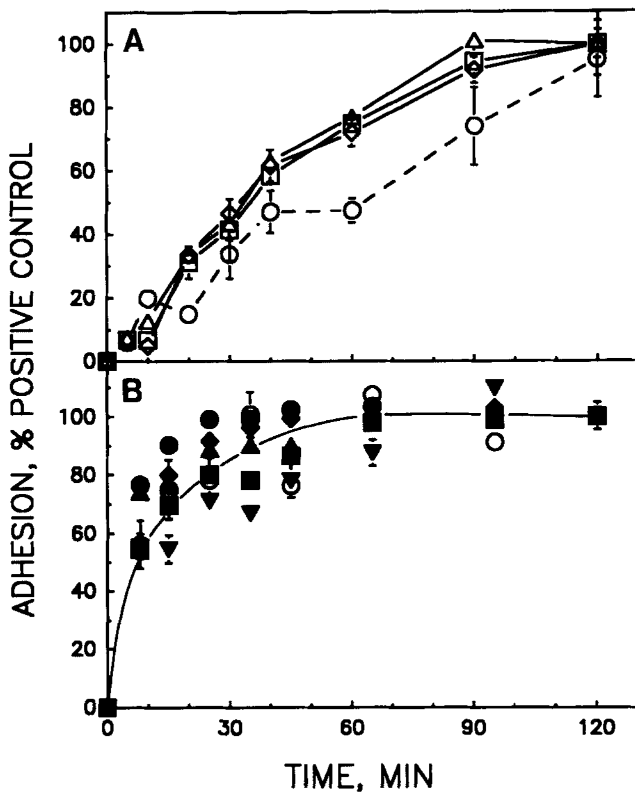

Figure 5. Kinetics of the adhesion of CHO wild-type and variant cells to Vn-coated substrata. Tissue culture plates were coated with 0.5 μg/ml (*A*) or 5 μg/ml (*B*) Vn and then blocked with BSA. CHO wild-type and variant cells were metabolically labeled with ^{35}S as described in Materials and Methods, washed, resuspended to a concentration of 10^5 cells/ml in α-MEM containing 1% BSA and 20 mM Hepes, pH 7.3, and allowed to adhere at 37°C for different time intervals. The attached cells were recovered and analyzed for radioactivity as described, and the number of adherent cells was calculated. The percent of total cells attaching during 120 min ranged from 50 to 90%; data were normalized using the number of cells attached at 120 min as 100%. In *A*: (○) wild-type cells; (△) clone 1-23; (□) clone 14-16; (◇) clone 21-4. In *B*: (○) wild-type cells; (●) clone B2; (▲) clone D1; (▼) clone 9; (■) clone 23; (◆) clone 25.

ate in some way to promote cell spreading on Vn or that FnR can recognize Vn under some circumstances and that this is necessary for fully developed cell spreading in CHO cells.

Cell Motility

The data shown in Fig. 7 indicate that there are major differences in the motile behavior of wild-type cells and FnR-deficient variants. There was virtually no movement of clones expressing ~2% levels of FnR from the upper to lower surfaces of the test filters. Clone 25, which expresses very low levels of FnR, was an outlier in this regard since some degree of cell motility was observed in this case. The basis of this difference is unclear at present; we are exploring the possibility that the clone 25 cells can use an adhesion factor other than Fn that may be present in small amounts in the assay system. The number of cells traversing the filters for clones expressing ~20% levels of FnR was 20–40% of wild type. These differences were not due to the initial adhesive behavior of the cells since cells of all clones could be observed to attach to the upper surfaces of the filters. The observed cell movements represent directed cell motility along an Fn gradient since, when Fn was present in both upper and lower chambers, only a few wild-type cells traversed the filter; we have not yet determined whether this directed movement represents chemotaxis or haptotaxis. It is remarkable that in the clones expressing ~20% of wild-type levels of FnR, impairment of directed cell motility in the presence of Fn was much more profound than impairment of cell adhesion (compare with Fig. 2).

Surface Labeling

To investigate the biochemical nature of the FnR deficiency at the level of the plasma membrane, we surface-labeled suspension-grown cells with $Na^{125}I$ and extracted the membrane proteins with a β-D-octylglucoside-containing buffer. Monoclonal antibody affinity supports were then used to selectively immunoprecipitate proteins expressing PB1 epitope or 7E2 epitope; SDS eluates of the washed supports were electrophoresed under nonreducing conditions, and the dried gels were subjected to autoradiography. Results for clones expressing ~20% of wild-type levels of FnR by ELISA are shown in Fig. 8, while results of clones expressing ~2% levels of FnR are shown in Fig. 9.

Purification with mAb PB1 (specific for the intact FnR) yielded two strongly labeled bands of about equal intensity from wild-type cells (Figs. 8 and 9, lanes *A*). Clones expressing 20% FnR based on ELISA displayed two weaker bands that comigrated with those from wild-type cells (Fig. 8, lanes *B–D*). Clones expressing 2% FnR displayed no labeled material, indicating essentially a complete lack of intact FnR on their surfaces (Fig. 9, lanes *B–F*). Although there is clearly a qualitative similarity between results obtained by surface labeling (Fig. 8) and by ELISA (Table I) for the 20% clones in that surface labeling is markedly reduced compared with wild type, there is not a good quantitative correlation between these two techniques. For example, clones 1-23 and 21-4 reproducibly give similar ELISA values but have quite different intensities of surface labeling. These discrepancies may simply be due to inherent differences in the abilities of these two techniques to reproducibly quantitate subtle alterations in surface antigen expression, with the ELISA assay likely to be more reliable.

Clones that express very low levels of FnR, either by ELISA (Table I) or by surface labeling and immunoprecipitation (Fig. 9, lanes *A–F*) using PB1 antibody, still express relatively high levels of 7E2 epitope (Table I), indicating continued presence of a β subunit on their surfaces. This could conceivably be due to surface expression of a "free" β chain or, perhaps more likely, to continued expression of β in association with another (non-FnR) α chain. Surface labeling and immunoprecipitation with 7E2 of clones expressing 2% FnR resulted in purification of two labeled bands (Fig. 9, lanes *H–L*). The faster component comigrated with authentic β subunit from wild-type cells, although it was reduced in intensity. A slower moving component was also observed (best seen in Fig. 9, lanes *H–J*) that seemed to migrate slightly ahead of FnR α chain (Fig. 9, lane *A*). This component (as well as some β chain) could also be observed in 7E2 immunoprecipitates from surface labeled lysates of wild-type cells exhaustively precleared with PB1 (data not shown). These results suggest that in clones that express only 2% of

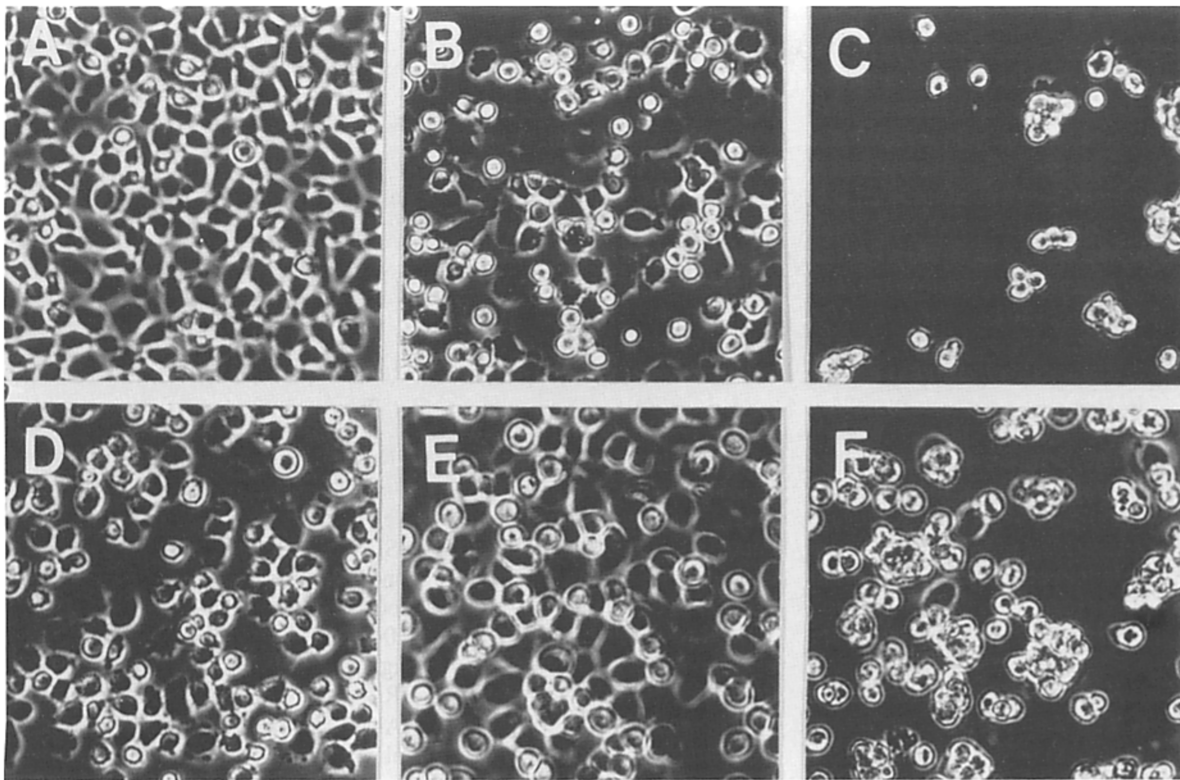

Figure 6. Morphologies of CHO wild-type and variant cells on Fn or Vn substrata. Washed CHO wild-type cells and variants (5×10^4 in 0.5 ml) in α-MEM containing 1% BSA and 20 mM Hepes, pH 7.3, were allowed to adhere for 30 min to 24-well tissue culture plate surfaces that had been precoated with either 1 μg/ml Fn (*A–C*) or 5 μg/ml Vn (*D–F*). Unattached cells were washed from the wells with α-MEM containing 1% BSA and 20 mM Hepes, pH 7.3, before the adherent cells were photographed using a Diaphot inverted phase photomicroscope (Nikon Inc., Garden City, NY). (*A* and *D*) Wild-type cells; (*B* and *E*) clone 1-23; (*C* and *F*) clone 25. A field with an unusually large number of attached cells is shown in *C* to allow comparison of morphology.

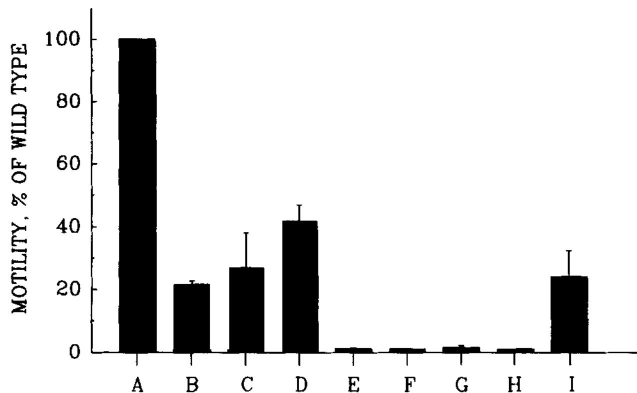

Figure 7. Comparison of motilities between CHO wild-type cells and FnR variant cells. NIH-3T3 fibroblast-conditioned medium supplemented with 30 μg/ml Fn was placed in the lower chamber of Boyden blind wells, and 3–5 $\times 10^5$ cells in a α-MEM containing 0.1% BSA was placed in the upper chamber. After 9 h, the blind wells were disassembled, the top side of the filter was cleaned, and cells on the bottom side were fixed, stained, and counted as described in Materials and Methods. The results are expressed as the percent of wild-type cells per microscopic field that have migrated to the lower side of the filter. The data presented are the composite of four experiments. (Bars *A–I*) Wild-type cells and clones 1-23, 14-16, 21-4, B2, D1, 9, 23, and 25, respectively.

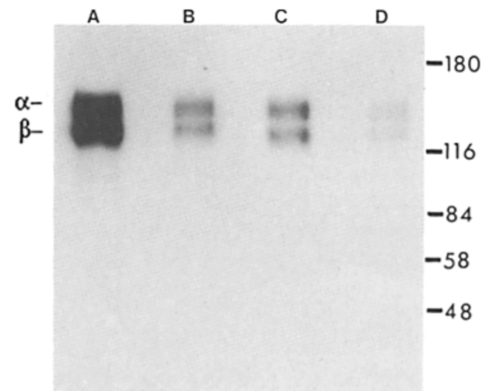

Figure 8. Electrophoretic analysis of affinity-purified receptor from 20% clones. Cells (2×10^7) were surface labeled with 400 μCi $Na^{125}I$, lysed in octyl glucoside-containing buffer, and incubated with PB1-Affigel. The Affigel was then washed, and bound material was eluted by addition of SDS sample buffer. Identical cell equivalents for each clone were loaded onto 7.5% SDS nonreducing gels along with prestained molecular mass markers. When the dye front reached the bottom of the gels, the gels were fixed in 0.3 M $CuCl_2$, dried, and autoradiographed. (Lanes *A–D*) CHO wild-type cells, clone 1-23, clone 14-16, and clone 21-4, respectively. The molecular masses of the standards to the right of the gel are in kilodaltons.

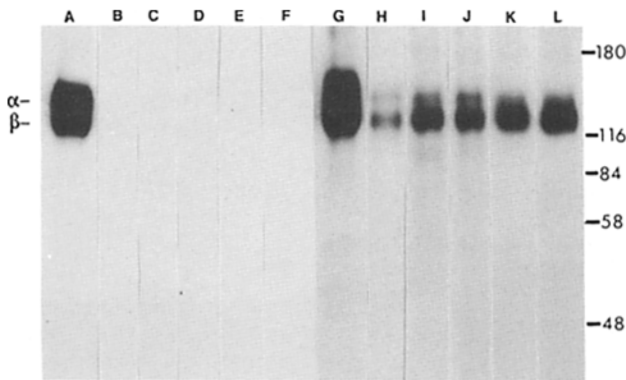

Figure 9. Electrophoretic analysis of affinity-purified receptor from 2% clones. Cells (4×10^7) were surface labeled with 800 μCi $Na^{125}I$ and lysed in octyl glucoside-containing buffer, and each lysate was divided into two parts. Half of each lysate was incubated with PB1-Affigel (lanes *A–F*) and half with 7E2-Affigel (lanes *G–L*). Receptor was eluted and electrophoresed, and the gel was autoradiographed as in Fig. 9. (Lanes *A–G*) CHO wild-type cells; (lanes *B* and *H*) clone B2; (lanes *C* and *I*) clone D1; (lanes *D* and *J*) clone 9; (lanes *E* and *K*) clone 23; (lanes *F* and *L*) clone 25. The molecular masses of the standards to the right of the gel are in kilodaltons.

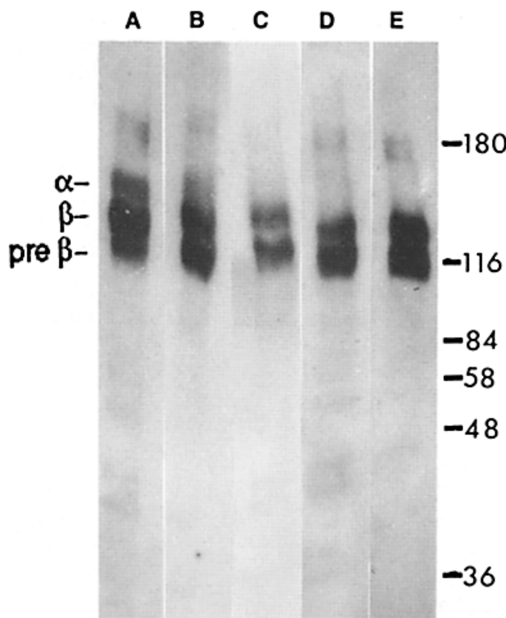

Figure 10. Immunoblot of CHO wild-type and variant clones. Identical cell equivalents (5×10^7) of each clone were washed and then lysed in octyl glucoside-containing buffer. FnR was partially purified by treating the lysates with wheat germ agglutinin-agarose affinity resin. The eluates in SDS sample buffer were electrophoresed on 7.5% nonreducing polyacrylamide gels along with molecular mass markers. At completion, the protein was transferred from the gel to a nitrocellulose filter, and the nitrocellulose was blotted with anti-gp140 goat polyclonal antibody (1 mg/ml) followed by rabbit anti-goat IgG (1:10,000 dilution) and ^{125}I-protein A (0.1 μCi/ml) with extensive washing in between each step; thereafter, the filter was dried and autoradiographed. The molecular masses of the standards are given in kilodaltons. (Lanes *A–E*) CHO wild-type cells and clones 1-23, B2, 23, and 25, respectively.

wild-type levels of FnR, a substantial amount of β chain continues to appear on the surface and that some of this is associated with a distinct, as yet unidentified, α chain. It is unlikely that this αβ heterodimer is itself an FnR since PB1 antibody, which is directed solely against the hamster homologue of $\alpha^5\beta_1$, can completely inhibit CHO cell adhesion to Fn. The autoradiograms of Fig. 9, lanes *H–L*, clearly show that relatively more surface labeling of β chain than α chain is seen in 7E2 immunoprecipitates of 2% clones, especially clones 23 and 25; this may result from preferential labeling of β vs. α chain. Alternatively, our results may suggest that some free β chain may emerge on the cell surface when expression of intact FnR is severely impaired. This observation contrasts somewhat with the more usual case where αβ assembly is a prerequisite for cell surface expression (1, 12, 27) but may be due to the major imbalance of α and β chain production.

Immunoblots

The lack of intact FnR on the cell surface in the variants may be due either to impairment of the biosynthesis of FnR subunits or to failure of those subunits to assemble properly. To sort out this problem, whole-cell lysates were immunoblotted with anti-FnR goat polyclonal antibody and ^{125}I-protein A; the results are shown in Fig. 10. Immunoblots of wild-type cells (Fig. 10, lane *A*) revealed three bands of labeled material—the α band, the β band, and a β subunit precursor at 140, 120, and ~110 kD—in agreement with previous observations (8). Clones from both populations (Fig. 10, lanes *B–E*) have bands that comigrate with β and with pre-β bands from wild-type lysate, while the α band was poorly detectable in 20% clones (Fig. 10, lane *B*) and not detected in 2% clones (lanes *C–E*). An interesting sidelight is that the relative amount of pre-β seems to be increased in all of the variants, thus suggesting that the maturation of pre-β is facilitated by the presence of α subunits, as other have reported (27).

RNA Blots

To determine if mRNA levels for the α subunit of FnR correlated with levels of FnR protein expression in wild-type and variant cells, we performed dot blot hybridizations with total RNA isolated from each clone. Fig. 11 shows that the levels of α chain mRNA parallel the levels of α chain and of intact FnR. Clone 1-23 (a 20% clone) has substantially less α chain mRNA than wild-type cells, while the α mRNA level in clone 25 (a 2% clone) was at background level. Parallel experiments with a β_1 chain cDNA probe showed equivalent levels of mRNA in each of these clones. This was typical of results from other clones in each group.

Discussion

Although there has been tremendous interest recently in the structure and function of integrins, our understanding of the role of these molecules in cell and tissue behavior is still in a nascent state. Much information on this topic can no doubt be gained by manipulating integrin function with antibodies or with peptide antagonists. Many insights will also be gained by expression of integrin cDNA constructs in ectopic situations; further, the use of antisense constructs may also permit

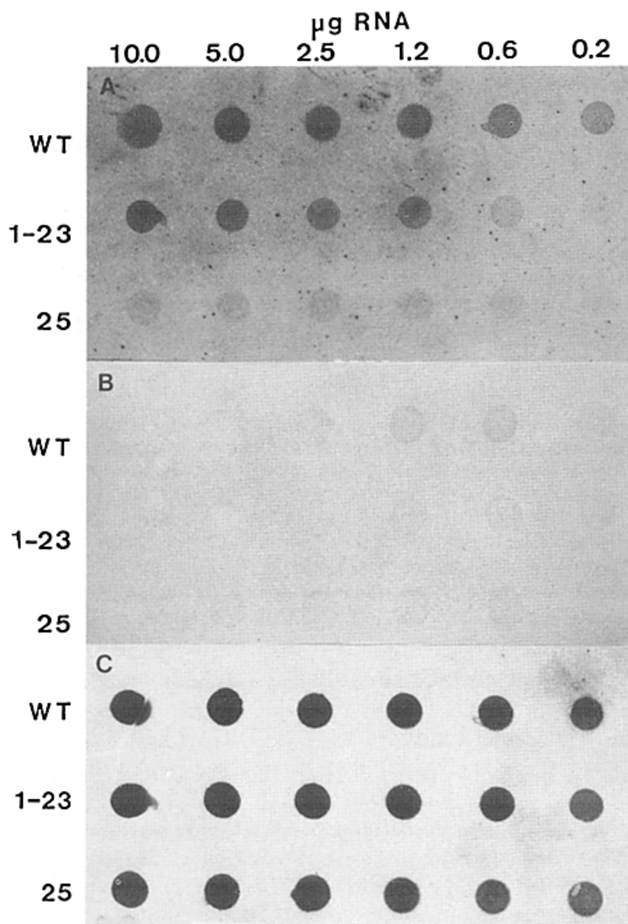

Figure 11. Dot blot analysis of total RNA from wild-type and variant cells. Serial dilutions of RNA from wild-type CHO cells and from clones 1-23 (20%) and 25 (2%) were loaded onto nitrocellulose and then hybridized with ^{35}S-labeled cDNA probes: (*A*) the 0.97-kb CHO FnR α chain probe; (*B*) the Bluescribe plasmid—a negative control; (*C*) a human β_1 cDNA.

studies involving the suppression of endogenous integrins. In addition to these approaches, however, the relatively straightforward selection of clonal cell populations deficient in integrin expression may also contribute significantly to elucidation of the multiple biological roles of integrin molecules. In this report we have described the selection and partial characterization of CHO cell clones deficient in expression of FnR, one of the most studied members of the integrin superfamily.

The first important point to emerge from these studies is that expression of FnR is not essential for growth and division of CHO cells. Thus, some clones that express $\leqslant$2% of wild-type levels of FnR, still display doubling times similar to wild-type cells when grown on serum-coated culture dishes. Presumably, the cells are able to use Vn or other adhesive factors in serum to maintain a relatively normal looking morphology and to permit growth. One should keep in mind that CHO cells are anchorage independent and thus may rely less on functions mediated by adhesion receptors than might be the case for anchorage-dependent cell lines. Other types of cells are known to express relatively low levels of $\alpha^5\beta_1$ and yet grow well (10, 13). However, it is interesting to note that the FnR can be lost from fibroblastic cells that normally express this molecule at high levels without impairment of cell replication.

A second significant point is that FnR abundance seems to be more critically involved in cell motility than in cell adhesion. Clones, such as 1-23 and 14-16, that possess ~20% of wild-type FnR levels seem to adhere quite well, albeit slightly more slowly, to Fn-coated surfaces and to assume an apparently normal morphology. This seems to support our previous finding that wild-type CHO cells have numerous spare receptors for Fn and that only a small fraction of the FnR population is used to form cell–substratum contacts (8). By contrast, cells with 20% of wild-type levels of FnR displayed marked impairments in a motility assay that involved directed migration in the presence of an Fn gradient. It may be that the cells' ability to locomote on Fn, which involves repetitive formation and breakage of adhesive contacts and possibly recycling of FnR (5), may have a more stringent requirement for abundant receptor than does simple formation of adhesive contacts. This may have implications in terms of the possible role of integrins in the invasive behavior of malignant cells; our results suggest that highly invasive cells may need abundant integrin receptors to locomote through extracellular spaces. Our findings are also generally consistent with observations on human leukocyte adhesion deficiency (LAD) (33–35). In LAD patients, leukocytes fail to produce normal β_2 chain and assemble β_2 integrins; these cells are markedly deficient in their ability to participate in control of infections or in inflammatory phenomena, an effect due in part to the inability of LAD cells to migrate from the bloodstream to sites of inflammation. Thus, cells may need to increase expression or mobilization of integrins for locomotion involved in tumor invasion, angiogenesis, or inflammatory phenomena.

A third interesting aspect of these studies is the observation that the FnR-deficient phenotype in all the clones isolated is due to reduced levels of FnR α mRNA and consequently reduced FnR α chain production. In wild-type cells, abundant α chain is detected by Western blotting with polyclonal anti–FnR, and α chain message is easily detected by dot blot with hamster α chain cDNA. In 2% clones neither α message nor polypeptide chain is detectable, while in 20% clones some message and approximately proportionate amounts of α chain polypeptide are found. The defects observed in our variants seem to be restricted to FnR since expression of VnR and ability to adhere to Vn is approximately normal in 20 and 2% clones; further, in 2% clones, with no FnR detectable by surface labeling, β chain partly associated with another α chain remains detectable. Failure to synthesize substantial amounts of FnR α chain results in underexpression of intact FnR at the cell surface; it also seems to result in accumulation of excess β chain precursor. This observation is consistent with those from other laboratories, suggesting that α chain synthesis is limiting and that β chain remains in precursor form until it combines with α (27) or is at least retarded in its maturation by the absence of α chain.

The nature of the defect leading to reduced levels of α chain mRNA is unclear at present. One possibility is an alteration in the 5′ regulatory sequence of the FnR α gene; other possibilities are a structural mutation leading to premature termination of transcription, production of unstable

message, or a major defect in splicing leading to products not recognized by our cDNA probes. We will attempt to discriminate among these possibilities in future experiments. A somewhat surprising aspect of our work is the fact that all of the variant clones studied thus far have a defect in α chain message levels. Thus far we have not identified any clones with a defect in β chain expression nor have we found variants that express aberrant but still recognizable α or β chains. This is somewhat surprising since 7E2, an anti-β chain monoclonal antibody, was used during the process of enrichment of cell populations for FnR-deficient variants. Further, the only known natural defect of the integrin superfamily, leukocyte adhesion deficiency (34), involves altered β chain. It may be that α chain message expression involves complex control mechanisms, thus permitting multiple opportunities for variant selection. If so, then further study of some of these variants, as well as the generation of additional variants, could lead to significant insights into the regulation of integrin expression.

An intriguing possibility for further work with these variants concerns their possible use as host cells for DNA transfection experiments. It may be that the defect that prevents accumulation of normal levels of endogenous α mRNA would also prevent efficient expression of α message produced from a transfection vector. If not, then these cells (especially the 2% variants) could be used to express normal or modified FnR cDNAs against a background essentially devoid of endogenous receptor; thus, both biochemical and functional studies could be readily conducted.

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