Insulin and Insulin-like Growth Factor-I (IGF-I) Receptor Overexpression in Breast Cancers Leads to Insulin/IGF-I Hybrid Receptor Overexpression: Evidence for a Second Mechanism of IGF-I Signaling[1]

Giuseppe Pandini, Riccardo Vigneri, Angela Costantino, Francesco Frasca, Antonio Ippolito, Yoko Fujita-Yamaguchi, Kenneth Siddle, Ira D. Goldfine, and Antonino Belfiore[2]

Istituto di Medicina Interna, Malattie Endocrine e del Metabolismo, Università di Catania, Ospedale Garibaldi, Catania, 95123 Italy [G. P., R. V., A. C., F. F., A. I., A. B.]; Department of Clinical Biochemistry, University of Cambridge, Addenbrookes' Hospital, Cambridge CB2 2QR, United Kingdom [K. S.]; Department of Molecular Biology, Beckman Research Institute of the City of Hope, Duarte, California 91010 [Y. F-Y.]; and Division of Diabetes and Endocrine Research, University of California, San Francisco, California 94115 [I. D. G.]

ABSTRACT

The insulin receptor (IR) form hybrids with the closely related insulin-like growth factor-I (IGF-I) receptor (IGF-I-R). Because most human breast carcinomas overexpress both the IR and the IGF-I-R, we evaluated whether the insulin/IGF-I hybrid receptor (Hybrid-R) is also overexpressed in these tumors and what role it plays in breast cancer biology.

Using specific ELISAs and Western blots, we measured Hybrid-R content and function in 8 human cultured breast cancer cell lines and 39 human breast cancer specimens. Hybrid-R content and function were also compared to the content and function of the IR and the IGF-I-R. Hybrid-R content exceeded the IGF-I-R content in >75% of breast cancer specimens and was directly related to the molar ratio of both the IR and IGF-I-R content, suggesting that Hybrid-R formation occurred by random assembly of IR and IGF-I-R half-receptors. Hybrid-Rs became tyrosine autophosphorylated when breast cancer cells were exposed to IGF-I but not when they were exposed to insulin.

In cells with an elevated Hybrid-R content, Hybrid-R autophosphorylation in response to IGF-I exceeded IGF-I-R autophosphorylation, suggesting that most of the IGF-I effect occurred via the Hybrid-R. Furthermore, Hybrid-Rs mediated growth in response to IGF-I, as indicated by experiments with blocking antibodies to the IGF-I-R.

These data indicated therefore that: (*a*) Hybrid-Rs are present and play a major role in mediating the IGF-I signal in breast cancer; (*b*) their expression is directly related to IR overexpression; and (*c*) potential therapies designed to block IGF-I actions in breast cancer must take into account the role of these Hybrid-Rs.

INTRODUCTION

Both the IR[3] and the closely related IGF-I-R are overexpressed in most human breast cancer specimens and cultured cells (1, 2), and both play a role in the biology of breast cancer (3, 4). When overexpressed, both the IR and the IGF-I-R induce a ligand-dependent transformed phenotype (5, 6), and their content in breast cancer correlates with clinical outcome (2, 7–9). The ligand for IGF-I-R, IGF-I, is produced by stromal fibroblasts in the cancer tissue and acts in a paracrine manner on the IGF-I-R of breast cancer cells (10, 11). In contrast, insulin, the ligand for the IR, is not locally produced. Thus, the exact role and hormone regulation of the IR in breast cancer are unknown.

Both the IR and the IGF-I-R are tetrameric complexes consisting of two identical extracellular α-subunits that bind the hormone and two identical β-subunits that have ligand-activated tyrosine kinase activity. In cells and tissues coexpressing both IR and IGF-I-R, Hybrid-Rs, formed by one IR α and one β-subunit hemicomplex and one IGF-I-R α and β-subunit hemicomplex, have been observed (12–14). Functional studies with purified Hybrid-Rs indicate that they behave like IGF-I-Rs rather than IRs because they bind IGF-I with an affinity similar to that of the IGF-I-R, whereas they bind insulin with a much lower affinity (14, 15).

Our understanding of the biological role of Hybrid-Rs, when compared to IRs and IGF-I-Rs, has been hampered in part

Received 8/27/98; revised 4/12/99; accepted 4/22/99.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by the Associazione Italiana per la Ricerca sul Cancro, Ministero dell'Università e della Ricerca Scientifica, e Technologica (M.U.R.S.T.) 60%, the Wellcome Trust, the J. A. Kerner Foundation, the Jay Gershow Cancer Fund, and the Ladies Auxiliary of the Veterans of the Foreign Wars. G. P., F. F., and A. I. were supported by fellowships from the Associazione Italiana per la Ricerca sul Cancro.

[2] To whom requests for reprints should be addressed, at Cattedra di Endocrinologia, University of Catanzaro, 88100, Policlinico Mater Domini, via T. Campanella, Catanzare, Italy. Phone: 39-95-32-62-90; Fax: 39-95-715-80-72; E-mail: belfiore@mbox.unict.it.

[3] The abbreviations used are: IR, insulin receptor; IGF-I, insulin-like growth factor-I; IGF-I-R, IGF-I receptor; Hybrid-R, IR/IGF-I-R hybrid receptor; PMSF, phenylmethylsulfonyl fluoride; CV, coefficient of variation.

Table 1 Characteristics of receptor monoclonal antibodies used

Antibody	Subunit	Epitope[a]	Binding to Hybrid-R[b]	Used in this study as[c]	Ref.
Anti-IR					
83-7	α		+	A, B	16
47-9	α		+	C	16
MA-20	α	489–559[d]	−	A, B	17
MA-51	α	485–599	−	C	18, 19
CT-1	β	1344–1355	+	D	20
Anti-IGF-I-R					
αIR-3	α	223–274	−	A, B, C	21, 22
17-69	α	440–586	+	A, B, D	23

[a] Amino acid residues (when known).
[b] As determined by ELISA.
[c] A, capturing antibody in ELISAs; B, immunoprecipitating antibody; C, blocking antibody in ligand stimulation studies; D, revealing antibody in ELISAs.
[d] I. D. Goldfine, unpublished observation.

by the difficulty of directly measuring the absolute content of these Hybrid-Rs. Currently, Hybrid-Rs have been measured as the proportion of ^{125}I-IGF-I binding immunoprecipitated by an anti-IR antibody (16). Thus, the presence, relative abundance, and functional role of Hybrid-Rs in human breast cancer are unknown.

In this report, we describe novel ELISAs that allow the direct measurement of the content of IRs, IGF-I-Rs, and Hybrid-Rs in breast cancer cells and specimens. These studies indicate that, in many breast cancers, the Hybrid-R is the major receptor that mediating the mitogenic response to IGF-I.

MATERIALS AND METHODS

The following materials were purchased as follows: MCDB-170 medium, FCS, bovine pituitary extract, and gentamicin obtained from Life Technologies, Inc. (Paisley, United Kingdom); and MEM, DMEM Nutrient Mixture-F-12 Ham's medium (1:1 DMEM/F-12), and all chemicals, unless otherwise specified, were purchased from Sigma Chemical Co. (St. Louis, MO). Tyr-A14-^{125}I-labeled insulin (specific activity, 13.3 MBq/μg) was provided by Dr. R. Navalesi (Dipartmento di Endocrinologia e Metabolismo, University of Pisa, Pisa, Italy). ^{125}I-labeled IGF-I (specific activity, 11.8 MBq/μg) was obtained from DuPont-NEN (Boston, MA). Antibodies to the IR and IGF-I-R and their reactivity with Hybrid-Rs are listed in Table 1. Monoclonal antibody αIR-3 was purchased from Oncogene Research (Cambridge, MA), anti-IGF-I-R α-subunit chicken polyclonal antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY), and anti-IR β-subunit polyclonal antibody was purchased from Transduction Laboratories (Lexington, KY). Antibodies MA-20, MA-51, CT-1, 83-7, 47-9, and 17-69 were obtained as described previously (Table 1; Refs. 16–23). Antiphosphotyrosine monoclonal antibody (αPy, clone 4G10) was purchased from Upstate Biotechnology, Inc.

MDA-MB231 and T47-D human breast cancer cell lines were provided by Dr. I. Perroteau (Dipartimento di Biologia Animale e dell'Uomo, University of Turin, Turin, Italy). MDA-MB157, MDA-MB453, MDA-MB468, and BT-20 breast cancer cells were provided by Dr. T. Kamalati (Sutton, London, United Kingdom). The immortalized 184B5 human mammary epithelial cell line was provided by Dr. M. R. Stampfer (Lawrence Berkeley National Laboratory Life Science Division, Berkeley, CA). MCF-7 and ZR-75-1 breast cancer cells and the immortalized human breast epithelial cells MCF-10 were obtained from the American Type Culture Collection (Manassas, VA).

Cell Lines and Human Tissue Specimens

Cell Lines. Breast cancer cell lines were grown in MEM supplemented with 2 mM glutamine, 10% FCS, nonessential amino acids, and 40 μg/ml gentamicin. The immortalized human epithelial cell lines MCF-10 and 184B5 cells were grown as described previously (24, 25). MCF-10 cells were cultured in DMEM/F-12 with 5% equine serum, 0.1 μg/ml cholera toxin, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, and 0.02 μg/ml epidermal growth factor. 184-B5 cells were cultured in MCDB-170 medium supplemented with 70 μg/ml bovine pituitary extract, 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 0.14 μM hydrocortisone, 0.1 mM ethanolamine, 0.1 mM phosphoethanolamine, and 5 μg/ml transferrin. In all cell lines, the medium was routinely changed every 2 days.

Normal Breast Tissue Isolation (Organoids). Normal breast tissue was isolated according to the organoid preparation protocol, as described previously (26). Briefly, breast tissue freshly obtained at reductive mammoplasty surgery from three subjects was cut into small pieces and washed twice with M199 medium containing 1000 units/ml penicillin, 1000 μg/ml streptomycin, and 25 ng/ml amphotericin B and incubated overnight at 4°C. Tissue samples were further cut into smaller pieces and incubated in M199 medium containing 200 units/ml collagenase, 160 units/ml ialuronidase, 0.5 μg/ml hydrocortisone, and 10 μg/ml insulin at 37°C for 24 h under continuous rotation. Organoids were then recovered by centrifugation and frozen in liquid nitrogen until processing.

Breast Tissue Specimens. Fresh tissue specimens were collected at surgery, carefully dissected by a pathologist to minimize contamination with stromal tissue, and immediately frozen and stored in liquid nitrogen until processing. Thirty-nine breast cancer specimens (32 ductal and 7 lobular carcinomas) were studied. In all, an adequate amount of tissue (~300 mg) for receptor measurements was available. Clinical information, including patient age and menopausal status, histopathological cancer characteristics, and tumor-node-metastasis stage were obtained by independent chart review. As controls, 9 breast fibroadenomas and 18 normal breast specimens were studied.

IR, IGF-I-R, and Hybrid-R Measurements

For receptor measurement in the ELISAs, cell monolayers or tissues were solubilized with 50 mM HEPES buffer (pH 7.6) containing 1 mM PMSF/1% Triton X-100 for 60 min at 4°C. The solubilized material was then centrifuged at 10,000 × *g*, and the supernatant was frozen at −80°C until assay. The protein content in the cellular extracts was measured by the bicinchoninic acid method (Pierce, Rockford, IL).

Purification of Standard IR, IGF-I-R, and Hybrid-R. The IR standard was purified by NIH-3T3 cells transfected with human IR cDNA, and the IGF-I-R standard was from CHO cells transfected with human IGF-I-R cDNA. Cells were solubilized with Triton X-100 and receptors purified by sequential affinity

chromatography on WGA-agarose and on agarose coupled either with the monoclonal antibody MA-20 (for IRs) or antibody αIR-3 (for IGF-I-Rs; Ref. 27). The Hybrid-R standard was obtained, as described previously (14), from human placental extracts by first immunocapturing Hybrid-Rs and IGF-I-Rs by a chromatographic step in αIR-3 antibody coupled agarose and then purifying Hybrid-R with a column containing agarose coupled to MA-51 antibody. This material was demonstrated to contain Hybrid-Rs by microsequencing, silver staining, and immunoblot (14). Receptor concentration was measured by amino acid analysis.

IR ELISA. IRs were captured by incubating cell or tissue lysates (0.5–60 μg/well) in Maxisorp immunoplates (Nunc, Roskilde, Denmark) precoated with 2 μg/ml anti-IR MA-20 antibody. After washing, the immunocaptured receptors were incubated with the biotinylated anti-IR CT-1 antibody [0.3 μg/ml in 50 mM HEPES-buffered saline (pH 7.6) containing 0.05% Tween 20, 1% BSA, 2 mM sodium orthovanadate, 1 mg/ml bacitracin, and 1 mM PMSF] and then with peroxidase-conjugated streptavidin. The peroxidase activity was determined colorimetrically by the addition of 100 μl of 3,3′,5,5′-tetramethylbenzidine [0.4 mg/ml in 0.1 M citrate-phosphate buffer (pH 5.0) with 0.4 μl/ml 30% H_2O_2]. The reaction was stopped by the addition of 1.0 M H_3PO_4, and the absorbance was measured at 451 nm.

IGF-I-R ELISA. IGF-I-Rs were measured by immunocapturing receptors with anti-IGF-I-R αIR-3 antibody and using biotinylated anti-IGF-I-R 17-69 as a second antibody. In both the IR and the IGF-I-R ELISAs, Hybrid-Rs (up to 1 ng/well) do not cause any appreciable interference.

Hybrid-R ELISA. Hybrid-Rs were measured by immunocapturing receptors with anti-IR 83-7 antibody and using biotinylated anti-IGF-I-R 17-69 as a revealing second antibody.

Coprecipitation of Receptor-^{125}I-Hormone Complexes. Assays were performed as described previously (16). Solubilized cells or tissues (20–40 μg of protein) were preincubated at 4°C with the radioactive ligand (20,000 cpm/50 μl for ^{125}I-insulin or 10,000 cpm/50 μl for ^{125}I-IGF-I; ~50–100 pM) in a final volume of 0.4 ml of 50 mM Tris-base buffer (pH 7.8) containing 200 kallikrein inhibitor units (KIU)/ml aprotinin, 1 mM *N*-ethylmaleimide, and 0.1% BSA for 20 h at 4°C before the addition of anti-IGF-I-R 17-69 or anti-IR 83-7 monoclonal antibody (10^{-8} M in 50 μl) for a further 1–2 h at 4°C. Antibody-bound radioactivity was determined using a sheep antimouse IgG adsorbent. Nonspecific binding was evaluated by including an excess (50–100 nM) of unlabeled hormones in the first incubation and was ~1 and 0.5% for ^{125}I-IGF-I and ^{125}I-insulin, respectively.

Total receptor-bound radioactivity was measured by precipitating the receptor-^{125}I-IGF-I or ^{125}I-insulin complexes with 0.1 ml of 0.4% bovine γ-globulin and 0.5 ml of 20% polyethylene glycol 6000 and allowing the mixture to precipitate for 20 min at 4°C. The supernatants were aspirated, and the radioactivity in the pellets was counted with a gamma counter. The radioactivity precipitated in the presence of excess unlabeled IGF-I (50 nM) or insulin (100 nM) was considered nonspecific binding.

Western Blot Analysis. Receptor subtypes were determined in six cancer cell lines by Western blot analysis by using aliquots of the same cell lysates prepared for ELISA (1 mg of total proteins).

IR measurement was performed by immunoprecipitating receptors with 5 μg of MA-20 monoclonal antibody and blotting with 1 μg/ml of an anti-IR β-subunit polyclonal antibody (Transduction Laboratories); IGF-I-R protein expression by immunoprecipitating receptors with 5 μg of αIR3 monoclonal antibody (Oncogene Research) and blotting with 1 μg/ml anti-IGF-IR α-subunit polyclonal antibody (Upstate Biotechnology, Inc.); Hybrid-R level was evaluated by immunoprecipitation with 5 μg of 17-69 monoclonal antibody and blotting with an anti-IR β-subunit polyclonal antibody (Transduction Laboratories).

Receptor Autophosphorylation

ELISA. IR, IGF-I-R, and Hybrid-R autophosphorylation was measured by ELISA. A procedure similar to that described for receptor measurement was used. Cells in monolayer cultures were stimulated with increasing doses (0–100 nM) or either insulin or IGF-I for 5 min at 37°C. Cells were then solubilized, and receptors were captured by incubation of cell lysates in Maxisorp plates precoated with various antireceptor antibodies (MA-20, αIR-3, 17-69, and 83-7). After washing, a biotinylated antiphosphotyrosine antibody [4G10, Upstate Biotechnology, Inc.; 0.3 μg/ml in 50 mM HEPES-buffered saline (pH 7.6), containing 0.05% Tween 20, 1% BSA, 2 mM sodium orthovanadate, 1 mg/ml bacitracin, and 1 mM PMSF] was added to reveal phosphorylated receptors by the peroxidase-conjugated streptavidin. The peroxidase activity was determined colorimetrically by the addition of 100 μl of 3,3′,5,5′-tetramethylbenzidine, and the absorbance was measured at 451 nm, as described above.

Western Blot Analysis. To evaluate Hybrid-R activation by either insulin or IGF-I, we carried out Western blot analysis in subconfluent cell monolayers serum starved for 24 h in 0.1% BSA-MEM and incubated in the presence or absence 10 nM insulin or IGF-I and 50 nM receptor subtypes blocking antibodies (αIR-3 for IGF-I-R; 47-9 for Hybrid-Rs) for 5 min at 37°C. Cells were then solubilized with 50 mM HEPES (pH 7.4), 1% Triton X-100, and 2 mM PMSF. Solubilized extracts were first IR immunodepleted by incubation with protein A-Sepharose coated with monoclonal antibody MA-20 and subsequently immunodepleted of typical IGF-I-Rs by incubation with Protein A-Sepharose coated with antibody αIR-3. The remaining Hybrid-Rs were immunoprecipitated with antibody 83-7. After centrifugation at 10,000 × *g* for 5 min, the pellets were washed three times at high stringency [0.5 M NaCl, 10 mM sodium phosphate (pH 7.4), 0.5% NP40, 2 mM EDTA, and 0.04% BSA], boiled in Laemmli buffer, and centrifuged at 10,000 × *g* for 5 min, and the supernatant was subjected to PAGE under reducing conditions. Proteins were transferred to nitrocellulose membranes that were then sequentially incubated with 1 μg/ml αPY and with a rabbit antimouse antiserum conjugated with horseradish peroxidase. The reaction was developed according to an enhanced chemiluminescence detection system (Amersham International, Amersham Place, United Kingdom).

To compare the autophosphorylation induced by IGF-I in typical IGF-I-Rs *versus* Hybrid-Rs, we exposed intact cell monolayers (MDA-MB157 or MCF-7) to IGF-I (10 nM). Cells were then solubilized, and receptors that were immunoprecipi-

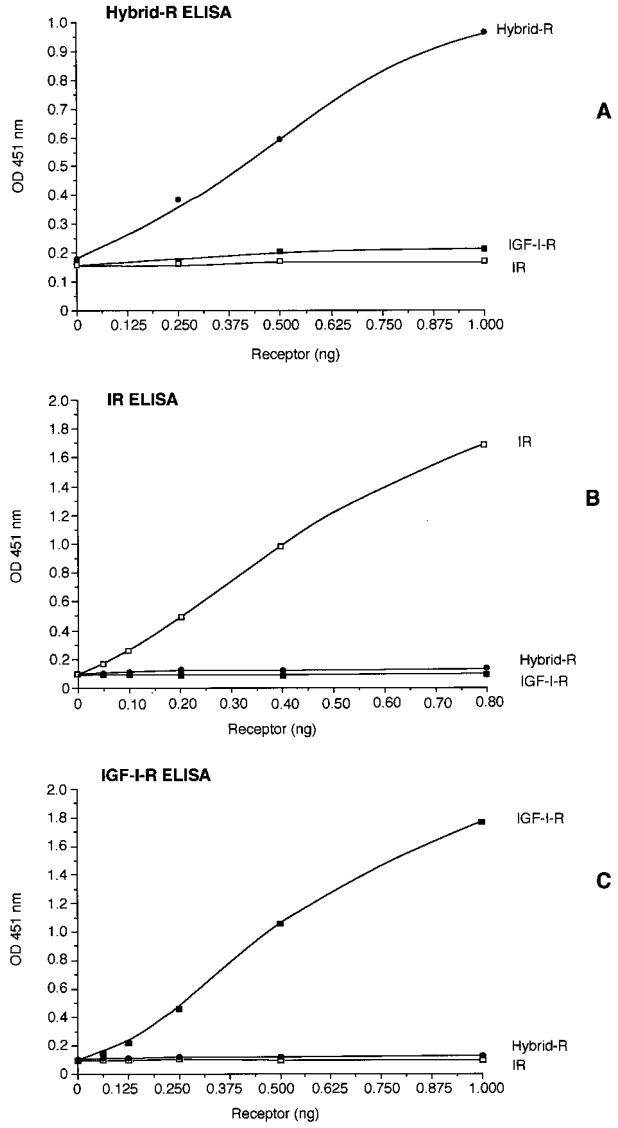

Fig. 1 Representative standard curves of ELISAs for Hybrid-Rs (*A*), IR (*B*), and IGF-I-R (*C*). There was no interference by the two other related receptors in any of the assays.

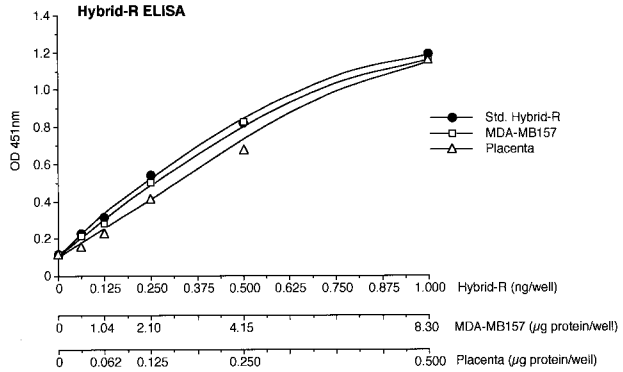

Fig. 2 A representative standard curve for the Hybrid-R ELISA is shown. Increasing concentrations of Triton X-100 extracts of both human placenta and MDA-MB157 breast cancer cells elicit dose-response curves that are parallel to those obtained with purified Hybrid-R standard.

tated with either αIR-3 or 83-7 antibodies were subjected to SDS-PAGE and immunoblotting with αPY, as described above.

To evaluate whether blocking antibodies to Hybrid-Rs inhibited IGF-I-R activation, we exposed MDA-MB157 or MCF-7 cell monolayers for 5 min to IGF-I (10 nM), together with either αIR-3 (antibody to IGF-I-R) or 47-9 (antibody to Hybrid-Rs). IGF-I-R and Hybrid-R autophosphorylation was then revealed by Western blot or by ELISA after immunoprecipitating or immunocapturing each receptor subtype with specific antibodies (αIR-3 and 83-7 for IGF-I-R and Hybrid-R, respectively).

Cell Growth Studies

To evaluate to what extent the mitogenic effect of IGF-I on breast cancer cells occurred via the IGF-I-Rs or the Hybrid-Rs, we measured the growth of MCF-7 or MDA-MB157 cells in the presence or the absence of various antireceptor blocking antibody (αIR-3 for IGF-I-R; 47-9 for Hybrid-Rs; MA-51 for IRs). Cells (5×10^3) were seeded in 96-well plates; after 24 h, the medium was removed and replaced with medium containing 2% charcoal-stripped FCS. After additional 24 h various concentrations of IGF-I (0–10 nM) were added in fresh medium with or without blocking antibodies. Cell growth was measured after 4 days, by measuring the rate of tetrazolium salts reduction to formazan, which is proportional to the number of living cells (EZ4U method; Biomedical, Wien, Austria). At the end of incubation, the absorbance was read at 450 nm.

In parallel experiments, cells were seeded in 25-mm multiwell plates at a density of 3×10^4 cells/well and incubated with IGF-I (with or without addition of antibodies) for 4 days with a medium change on day 3. Cells were detached with a 0.2% EDTA solution and counted in a hemochromocytometer. The cellular suspension was then centrifuged, the pellet was solubilized with 0.03% SDS, and the cellular DNA content was determined by a fluorimetric method.

Statistical Analysis

IR, IGF-I-R, and Hybrid-R cell and tissue contents were correlated by the Spearman rank correlation or by linear regression analysis after data transformation into natural logarithmics. Correlation of Hybrid-Rs with other variables was carried out by logistic regression. The statistical package SPSS (SPSS Inc., Chicago, IL) was used.

RESULTS

Measurements of Hybrid-Rs, IR, and IGF-I-R

Specific ELISAs. A variety of monoclonal antibodies to the IR and the IGF-I-R were screened for use in the Hybrid-R ELISA (Table 1). A sensitive and specific assay was developed by first capturing the Hybrid-R with IR α-subunit antibody 83-7 and reading out with biotinylated IGF-I-R α-subunit antibody 17-69 (Fig. 1*A*). The minimal detectable content of Hybrid-Rs was 0.125 ng/well (1.25 ng/ml). Neither purified IR nor purified

Table 2 Proportion of Hybrid-Rs, as measured by both ELISA and coprecipitation assay methods

Breast cells	Proportion of Hybrid-R (%)	
	ELISA[a]	Coprecipitation[b]
Nonmalignant		
MCF-10	56.3	47.8
184B5	32.3	28.5
Malignant		
T47-D	27.5	36.4
BT-20	28.3	34.1
MCF-7	22.7	12.7
MDA-MB231	69.8	55.6
ZR-75	47.4	60.2
MDA-MB157	85.7	87.2
MDA-MB468	89.3	96.3
MDA-MB453	88.6	78.7

[a] Hybrid-R/(IGF-I-R + Hybrid-R) × 100.
[b] % of total ^{125}I-IGF-I binding that was precipitated by an anti-IR antibody.

Table 3 IR, IGF-I-R, and Hybrid-R content, as measured in cultured breast cells by specific ELISAs

Predicted Hybrid-R content is calculated on the assumption of random assembly on the basis of IR and IGF-I-R cell content.

Breast cells	Content of receptor (ng/100 μg protein)			
	Measured IR	Measured IGF-I-R	Measured Hybrid-R	Predicted[a] Hybrid-R
Nonmalignant				
MCF-10	0.7	2.2	2.8	2.5
184B5	1.1	1.2	2.0	2.3
Malignant				
T47-D	0.4	10.0	3.8	4.0
BT-20	0.5	7.6	3.0	3.9
MCF-7	1.0	34.0	10.0	11.6
MDA-MB231	2.6	2.6	6.0	5.1
ZR-75	5.0	10.0	9.0	14.1
MDA-MB157	11.0	2.0	12.0	9.2
MDA-MB468	15.0	1.2	10.0	8.6
MDA-MB453	16.0	1.8	14.0	10.8

[a] If concentration of IR monomer is *I*, and that of IGF-I-R monomer is *G*, then, for random assembly, expected ratios are: IR(*II*):IGF-I-R (*GG*):Hybrid-Rs (*IG*) = I^2:G^2:2*IG*. Therefore, predicted concentrations of Hybrid-Rs = $2\sqrt{\text{IR}}\sqrt{\text{IGF-I-R}}$.

IGF-I-R reacted in the assay (Fig. 1*A*). Moreover, there was no interference from the ligands, insulin, or IGF-I (data not shown). Multiple dilutions of extract of MDA-MB157 breast cancer cells and placenta tissue produced dose-response curves that paralleled those obtained with purified Hybrid-R standard (Fig. 2). Intra-assay CVs were <7% at 0.5 ng/tube and <8% at 1.0 ng/tube. Inter-assay CVs were <8 and <10%, respectively.

The ELISAs used for IR and IGF-I-R had similar features of sensitivity and specificity (Fig. 1, *B* and *C*, respectively). The minimal detectable content of IR was 0.05 ng/tube, and the minimal detectable content of IGF-I-R was 0.0625 ng/tube. For both assays, the intra-assay CVs were <8%, and interassay CVs were <10%.

Comparison with the Coprecipitation Assay. To compare the results obtained by this new Hybrid-R ELISA with the older coprecipitation method for Hybrid-R (16), we compared eight breast cancer cell lines and two nonmalignantly transformed cell line. With the coprecipitation method, the Hybrid-R content was evaluated as the proportion of total ^{125}I-IGF-I binding (a measure of both IGF-I-Rs and Hybrid-Rs) that was immunoprecipitated by a monoclonal antibody (Ab 83-7) that recognized the Hybrid-R but not the IGF-I-R (16). When the proportion of Hybrids-R was calculated by ELISA and compared to the proportion of Hybrid-Rs calculated by the coprecipitation method, the results were nearly identical ($r = 0.916$, $P = 0.001$, Spearman rank correlation; Table 2). In breast cancer cells, Hybrid-Rs represented 12–96% of receptors interacting with IGF-I.

IR, IGF-I-R, and Hybrid-R Content in Human Breast Cancer Cells by ELISA and Western Blot

The IR, IGF-I-R, and Hybrid-R contents were first measured in a panel of cultured human epithelial breast cells (Table 3). In breast cancer cell lines, the IR content ranged from 0.4 to 16.0 ng/100 μg protein, and IGF-I-R ranged from 1.2 to 34.0 ng/100 μg protein. In these cell lines, there was no significant correlation between IR and IGF-I-R content. In some cell lines (T47-D, BT-20, and MCF-7), the IGF-I-R content was 15–30-fold higher than IR content. In other cells (MDA-MB157, MDA-MB468, and MDA-MB453), the opposite was observed, with the IR content being 5–12-fold higher than IGF-I-R content. In two breast cancer cell lines (MDA-MB231 and ZR-75), levels of IR and IGF-I-R content were similar.

The cellular content of Hybrid-Rs ranged from 2.8 to 14.0 ng/100 μg protein and was higher than the IGF-I-R content in all cell lines in which the IR content exceeded or was similar to the IGF-I-R content (Table 3). In nonmalignant breast cells (MCF-10 and 184-B5), the content of all three receptor types was relatively low (Table 3).

The value for Hybrid-R content predicted on the basis of random assembly of cell IR and IGF-I-R half-receptors[4] correlated very closely with the measured cellular content of Hybrid-R ($r = 0.810$, $P = 0.008$).

IR, IGF-I-R, and Hybrid-R were also measured in six breast cancer cell lines by Western blot analysis; receptor contents were very close to those found by ELISA (Fig. 3).

Effect of Variation of IR Content on Hybrid-R Content

To evaluate how changes of cell IR content may affect Hybrid-R content, we investigated the U937 human myeloid cell line. These cells have a relatively high IR content, which decreases by ~90% (from 5.3 ng/100 μg protein to 0.6 ng) when they differentiate into macrophages after treatment with 12-*O*-tetradecanoylphorbol-13-acetate. After 12-*O*-tetradecanoylphorbol-13-acetate treatment, the Hybrid-R content decreased from 3.8 to 1.5 ng, a value in close accordance with the values

[4] If the total concentrations of IR and IGF-I-R half-receptors are *I* and *G*, respectively, and these half receptors combine randomly, then it would be predicted that the relative concentrations of IR:IGF-I-R: Hybrid-Rs would be I^2:G^2:2*IG*. Thus, the measured content of Hybrid-Rs can be compared with the expected on the basis of random assembly because Hybrid-Rs = $2\sqrt{\text{IR}}\sqrt{\text{IGF-I-R}}$.

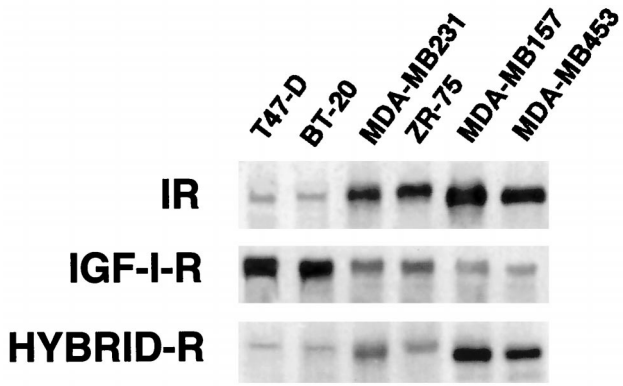

Fig. 3 Western blot of IR, IGF-I-R, and Hybrid-R proteins in six human breast cancer cell lines. Subconfluent monolayers were solubilized, and proteins were subjected to immunoprecipitation by using MA-20 monoclonal antibody for IR, αIR-3 monoclonal antibody for IGF-I-R, and 17-69 monoclonal antibody for Hybrid-R. Filters were blotted using an anti-IR β-subunit polyclonal antibody to detect IR and Hybrid-R and an anti-IGF-I-R α-subunit polyclonal antibody to detect IGF-I-R. A representative of three experiments is shown.

predicted by the random assembly model. IR overexpression, therefore, contributes to an increased Hybrid-R formation and to the signaling and biological effects of IGF-I.

IR, IGF-I-R, and Hybrid-R Content in Human Breast Cancer Tissue Specimens

The content of IRs, IGF-I-Rs, and Hybrid-Rs were then measured by ELISA in 39 breast cancer specimens, 9 breast fibroadenomas, and 18 normal breast specimens (Table 4). As reported previously (1, 2), the average content of both IR and IGF-I-R was significantly higher ($P < 0.0001$) in cancer tissues than in normal tissues and fibroadenoma tissue (Table 4). In addition, fat-free normal breast tissue was obtained by preparing organoids from three normal breast specimens obtained by reductive mammoplasty (26). Receptor content in normal breast tissue was very similar when measured either in fat-free organoids or in whole tissue lysates, indicating that the low receptor content measured in normal breast was not due to the contribution of adipose tissue (Table 4).

In 39 breast cancer specimens, the Hybrid-R content was ~3-fold greater than that of either IR or IGF-I-R. Hybrid-R content exceeded the IGF-I-R content in 30 cases. In all 39 breast cancer specimens, Hybrid-R content was significantly related to both IR ($r = 0.618$, $P = 0.0001$) and IGF-I-R content ($r = 0.576$, $P = 0.0001$, Spearman rank correlation), whereas the contents of IR and IGF-I-R were not correlated. Receptor content in benign breast tumors was intermediate between normal and cancer tissue but closer to that found in normal breast.

No significant association was found between cancer Hybrid-R content and the following variables: tumor estrogen or progesterone receptor content, tumor-node-metastasis stage, patient age or menopausal state, or presence of peritumoral lymphocytic infiltration. Also in tissue specimens, the measured Hybrid-R values significantly correlated with values predicted on the basis of the random assembly model ($r = 0.735$, $P = 0.0001$). Hybrid-R content was correlated with both IR ($r = 0.69$, $P = 0.0001$) and IGF-I-R content ($r = 0.75$, $P = 0.0001$) also in normal breast and in fibroadenomas.

Table 4 IR, IGF-I-R, and IR/IGF-I-R hybrid content (ng/100 μg protein) in breast cancer specimens, breast fibroadenomas, and normal breast specimens

	IR	IGF-I-R	IR/IGF-I-R
Breast cancer tissue ($n = 39$)			
Mean ± SD	4.50 ± 5.37	3.67 ± 6.57	6.04 ± 4.67
Range	0.2–25.0	0.3–39.5	0.8–28.0
Median	2.20	1.90	6.01
Fibroadenomas ($n = 9$)			
Mean ± SD	2.23 ± 1.64	0.74 ± 0.35	2.33 ± 1.92
Range	0.4–5.0	0.2–1.3	1.0–6.9
Median	1.55	0.78	1.60
Normal breast ($n = 18$)			
Normal breast specimens ($n = 18$)			
Mean ± SD	1.0 ± 1.1	0.50 ± 0.31	1.12 ± 0.87
Range	0.1–4.8	0.1–1.2	0.1–2.8
Median	0.65	0.41	0.77
Organoids ($n = 3$)			
Mean ± SD	0.94 ± 0.04	0.84 ± 0.28	0.88 ± 0.26
Range	0.89–0.98	0.55–1.1	0.6–1.1
Median	0.95	0.87	0.95

Hybrid-R, IGF-I-R, and IR Autophosphorylation in Breast Cancer Cells in Response to IGF-I or Insulin (by ELISA and Western Blot)

We then studied Hybrid-R tyrosine kinase activity in breast cancer cells after stimulation by either insulin or IGF-I or both. MDA-MB157 cells were stimulated with either insulin or IGF-I and lysed, and receptor autophosphorylation was evaluated by ELISA after capture with four specific monoclonal antibodies (Fig. 4). When IGF-I-Rs were immunocaptured with the monoclonal antibody αIR-3, IGF-I but not insulin induced a dose-dependent receptor autophosphorylation (EC_{50} = 0.2 nM; Fig. 4*A*). In contrast, when IR were captured with monoclonal antibody MA-20, an autophosphorylation occurred in response to insulin (EC_{50} = 0.5 nM; Fig. 4*B*) but not in response to IGF-I. Hybrid-Rs were immunocaptured with two different antibodies (the anti-IR antibody 83-7 and the anti-IGF-I-R antibody 17-69). As expected, IGF-I stimulated autophosphorylation in both conditions (EC_{50} = 0.35–0.5 nM; Fig. 4, *C* and *D*), whereas insulin stimulated autophosphorylation only when IRs were present (immunocapturing antibody, 83-7; Fig. 4*C*).

Western blot analysis of Hybrid-R autophosphorylation was also carried out in MDA-MB157 cells stimulated with either IGF-I or insulin. Cell extracts were first immunodepleted of both typical IRs and IGF-I-Rs using antibodies MA-20 and αIR-3, and then Hybrid-Rs were immunoprecipitated with antibody 83-7. As expected, Hybrid-Rs were autophosphorylated in response to IGF-I but not to insulin (data not shown). These data indicate that, as shown previously for isolated receptors (15), in intact breast cancer cells, Hybrid-Rs function as IGF-I-Rs.

Biological Effect of Hybrid-R Stimulation by IGF-I

Receptor Autophosphorylation and Blockade by Monoclonal Antibodies. To evaluate the significance of Hybrid-R in mediating the biological effects of IGF-I, we studied both

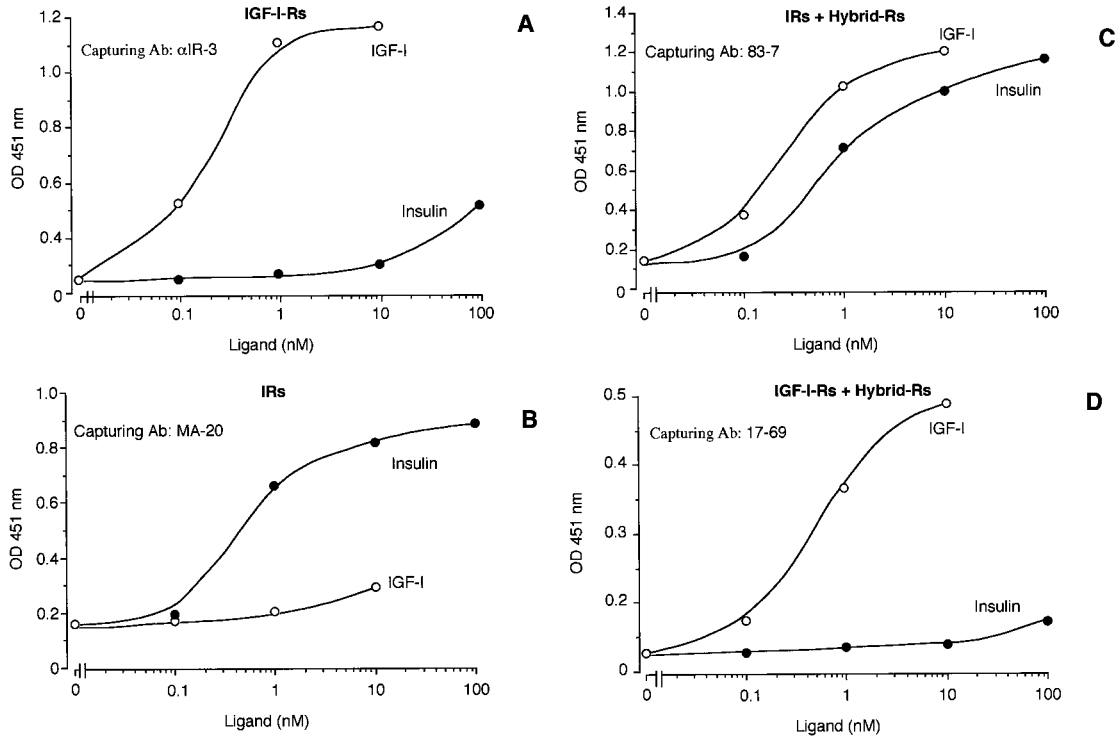

Fig. 4 IR, IGF-I-R, and IR/IGF-I-R autophosphorylation in intact cells. Cells were exposed for 5 min to increasing concentrations of insulin or IGF-I and than solubilized. Receptors were immunocaptured with: αIR-3 (an anti-IGF-I-R antibody that reacts poorly with Hybrid-Rs, *A*); MA-20 (an anti-IR antibody that does not recognize Hybrid-Rs, *B*); 83-7 (an antibody that recognizes both IR and Hybrid-Rs, *C*); 17-69 (an antibody that recognizes both IGF-I-R and Hybrid-Rs, *D*).

MCF-7 and MDA-MB157 breast cancer cells, each of which has a different IGF-I-R:Hybrid-R ratio. Cells were first exposed to IGF-I (10 nM), and either the IRs, IGF-I-Rs, or Hybrid-Rs were immunoprecipitated, subjected to SDS-PAGE, and blotted with anti-PY antibody (Fig. 5). IGF-I-stimulated autophosphorylation of IGF-I-Rs was high in MCF-7 cells but relatively low in MDA-MB157 cells. In contrast, IGF-I stimulated Hybrid-R autophosphorylation was relatively low in MCF-7 cells but relatively high in MDA-MB157 cells. These data were in close accordance with the different IGF-I-R:Hybrid-R ratios in these cell lines (Table 2).

Receptor autophosphorylation was then measured in cells exposed to 10 nM IGF-I in the presence or absence of blocking antibodies that were specific for either the IGF-I-R (αIR-3) or Hybrid-R + IR (Ab 47-9). As expected, blockade with αIR-3 markedly inhibited IGF-I-R activation but not Hybrid-R activation. In contrast, Ab 47-9 inhibited Hybrid-R activation (Fig. 6, *A* and *B*). The latter effect was more evident in MDA-MB157 cells, in which Hybrid-Rs represented the majority of IGF-I binding sites.

Mitogenesis after Blockade by Monoclonal Antibodies. We then evaluated the effect of receptor blockade on cell growth. IGF-I stimulation of growth was most strongly inhibited by Ab 47-9 (Hybrid-R blocking antibody) in MDA-MB157 cells, in which Hybrid-Rs represent the majority of IGF-I binding sites (Fig. 7*A*). In contrast, IGF-I stimulation of growth was most strongly inhibited by αIR-3 (IGF-I-R blocking antibody)

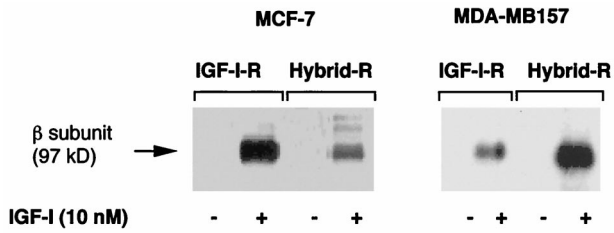

Fig. 5 Western blot analysis of IGF-I-Rs and Hybrid-Rs autophosphorylation in MCF-7 and MDA-MB157 cells. After exposure to IGF-I, IGF-I-Rs were immunoprecipitated with αIR-3, and Hybrid-Rs were immunoprecipitated with 83-7 antibody. Phosphorylated receptor were detected by an anti-PY antibody.

in MCF-7 cells, in which IGF-I-Rs represent the majority of IGF-I binding sites (Fig. 7*B*). As expected, blockade of IRs by antibody MA-51 did not affect IGF-I-stimulated growth in either cell type.

DISCUSSION

We previously reported that the IR and the related receptor IGF-I-R are overexpressed in breast cancers (1, 3) and that both the IR and IGF-I-R are expressed at high levels in 45% of these cancers. Interestingly, IR content exceeds IGF-I-R content in ~40% of cases, whereas IGF-I-R content exceeds IR content in 15% of cases (7). Using immunohistochemistry, we observed

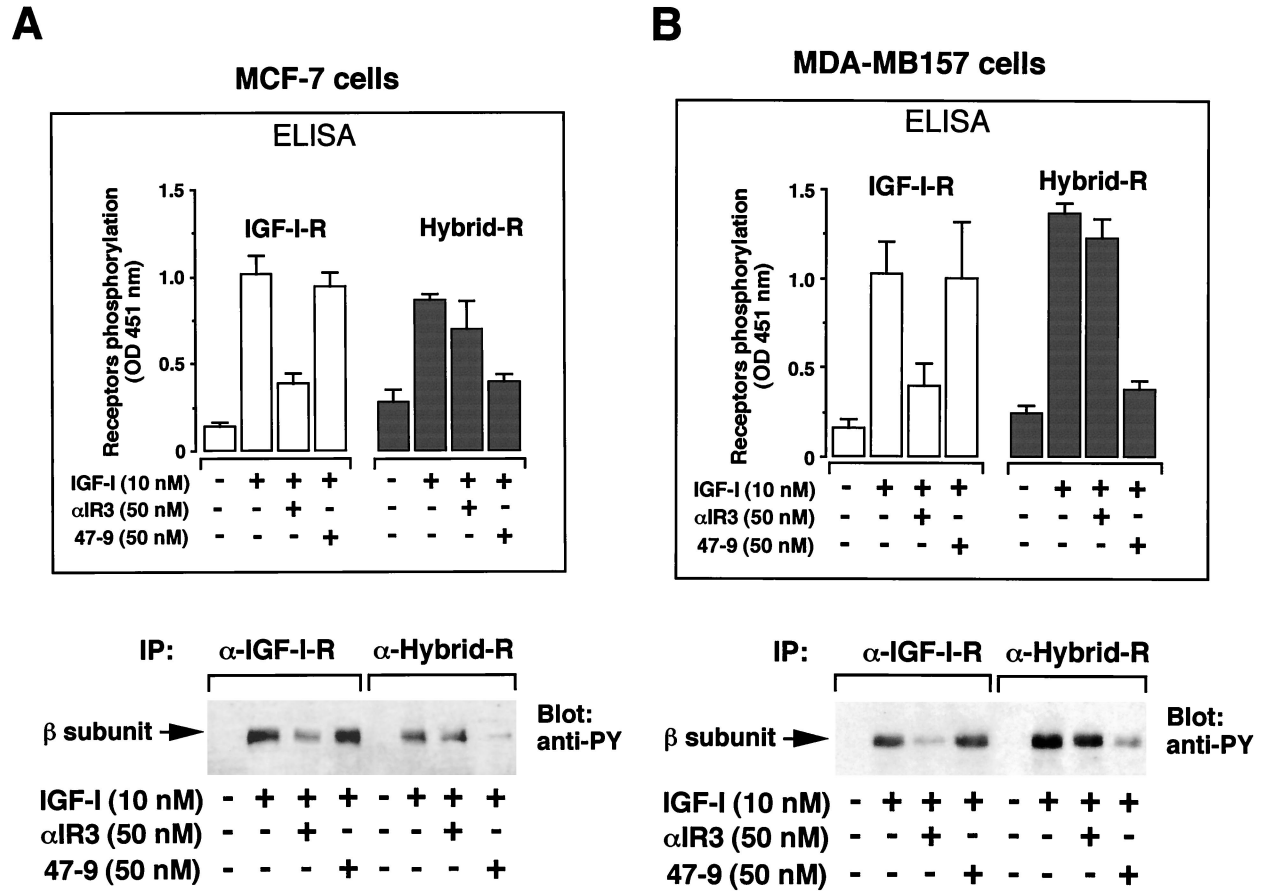

Fig. 6 Blockade of IGF-I-R and Hybrid-R autophosphorylation in MCF-7 (*A*) and MDA-MB157 (*B*) cells by competitive selective antibodies. Serum-starved subconfluent cell monolayers were exposed to 10 nM IGF-I for 5 min in the presence or absence of either IGF-I-R-blocking antibody αIR-3 or Hybrid-R-blocking antibody 47-9 (50 nM). Receptor phosphorylation in cell lysates was revealed by ELISA (*top*) and Western blot (*bottom*). αIR-3 was used to immunopurify IGF-I-R, and 83-7 was used to immunopurify Hybrid-Rs. Phosphorylated receptors were detected using antiphosphotyrosine antibody (biotin-conjugated in ELISAs). *Top*: *columns*, means of three separate experiments; *bars*, SE. *Bottom*, representative experiments.

that IRs were overexpressed by the neoplastic epithelial cells and that the content of IR in these cells was related to clinical outcome (1, 9). IR expression was a major predictor of reduced disease-free survival in lymph node-negative breast cancers (9). Because insulin is not locally produced in breast cancer tissues, it was not known by what mechanism the IR was activated, thus leading to altered cellular behavior.

There are several lines of evidence that the growth factor IGF-I plays a major role in breast cancer. (*a*) It is a potent transforming and mitogenic agent (11). (*b*) It is locally produced by breast cancer stromal cells (10). (*c*) Its receptor, the IGF-I-R, is overexpressed in most breast cancer cells. (*d*) IGF-I-R expression is an obligatory requirement both for developing a transformed phenotype and protecting cancer cells from apoptosis (28). For these reasons, the IGF-I/IGF-I-R ligand-receptor paracrine loop has been the target of a variety of strategies aiming to inhibit IGF-I signaling in cancer cells (29–31). In contrast, the IR binds IGF-I with low affinity and, thus, does not participate in this loop.

In addition to homotypic IRs and IGF-I-Rs formed by identical αβ half-receptors that are disulfide-linked into $\alpha_2\beta_2$ tetramers, the existence of heterotypic Hybrid-Rs has been shown in a variety of human tissues and cultured cells expressing both IR and IGF-I-R (32). Hybrid-Rs are heterotetrameric complexes that are formed because a proportion of IR αβ half-receptors assemble with IGF-I-R αβ half-receptors, given the high degree of homology between these two receptors. Assembly of αβ half-receptors into either homotypic IRs and IGF-I-Rs or Hybrid-Rs appear to result from a random process (32): a high proportion of Hybrid-Rs results, therefore, from the expression of a large excess of one of the two homotypic receptors (33).

Purified Hybrid-Rs bind ~20 times as much IGF-I than insulin, and IGF-I has a greater ability than insulin to stimulate Hybrid-R autophosphorylation, which occurs by intramolecular *trans*-phosphorylation of both β-subunits (33, 34). These studies suggest, therefore, that Hybrid-Rs behave like IGF-I-Rs, although their functional importance in both normal and neoplastic tissues is not known.

Herein, we document that IR overexpression in breast

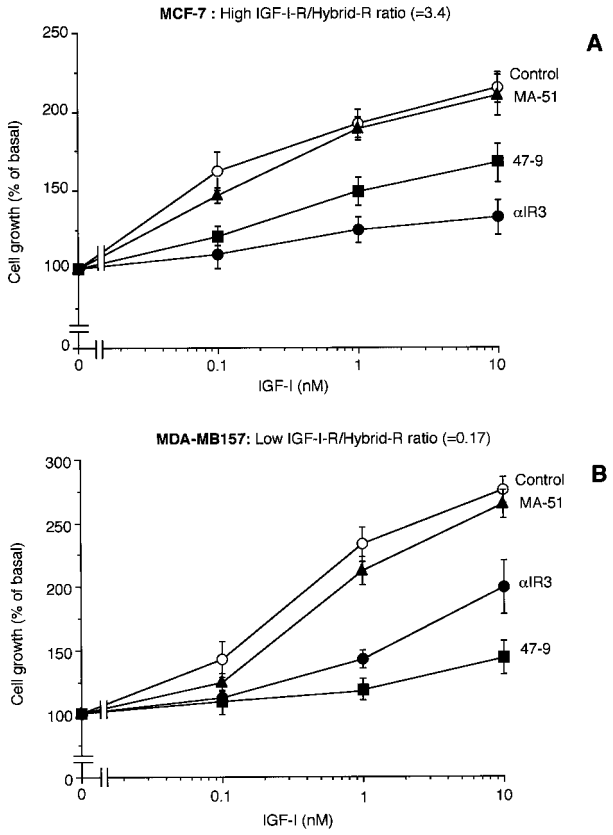

Fig. 7 IGF-I-induced growth in MCF-7 cells (*A*) or MDA-MB157 cells (*B*) in the presence of either an unrelated antibody (control) or antibodies inhibiting either the IR (MA-51), the IGF-I-R (αIR-3), or Hybrid-Rs (Ab 47-9). Cell growth was mostly inhibited by blocking the IGF-I-R in MCF-7 cells, whereas it was mostly inhibited by blocking Hybrid-R in MDA-MB157 cells.

cancers is associated with an increased content of Hybrid-Rs. Because Hybrid-Rs bind IGF-I but not insulin, they behave as functional IGF-I-Rs. IR overexpression and the consequent increased formation of Hybrid-Rs, therefore, produces an additional functioning receptor that responds to IGF-I. Moreover, we now find that, in most breast cancer and tissues, a considerable proportion of the mitogenic effects of IGF-I can be inhibited by blocking the binding of IGF-I to Hybrid-Rs. Thus, the Hybrid-R plays a major role in IGF-I signaling.

Previously, the relative roles of the IR, IGF-I-R, and Hybrid-R were difficult to assess because of the structural and functional homology between these receptors and the lack of assays to directly measure Hybrid-R content. Using a newly developed ELISA, we are now able to directly measure the content of the Hybrid-R in addition to that of the IR and the IGF-I-R. Moreover, in breast cancer cells, we measured the autophosphorylation of these receptors in response to either IGF-I or insulin. We confirm that IGF-I activates the Hybrid-R with an affinity similar to that of the IGF-I-R (15). Moreover, in cultured cells, prevalent receptor autophosphorylation after exposure to IGF-I is closely related to the relative abundance of either IGF-I-R or Hybrid-Rs.

Finally, we studied the effect of monoclonal antibodies specific to either Hybrid-R or IGF-I-R on the mitogenic effect of IGF-I. Cell growth studies in the presence of these antibodies indicated that, in a given cell type, the IGF-I mitogenic effect occurred predominantly via the receptor type that was more abundant. Thus, an anti-IR antibody that recognized the Hybrid-R (Ab 47-9) substantially blocked growth in breast cancer cells that had a high Hybrid-R:IGF-I-R ratio. In contrast, an anti-IGF-I-R antibody that recognizes the IGF-I-R (αIR-3) substantially blocked growth in breast cancer cells that had a high IGF-I-R:Hybrid-R ratio.

In summary, these data provide new insights into the role of IR overexpression in breast cancer by demonstrating that IR overexpression increases the cellular response to IGF-I by increasing Hybrid-R formation. Moreover, in light of these data, a reconsideration of therapeutic strategies for breast cancer based on the blockade of the IGF-I/IGF-I-R loop may be needed. A more effective receptor-based strategy may occur when both the IGF-I-R and the IR are chosen as targets for down-regulation. Furthermore, a more effective blockade of IGF-I signaling may be obtained by using either antibodies or other agents that block IGF-I binding to both Hybrid-Rs and the IGF-I-Rs.

REFERENCES

1. Papa, V., Pezzino, V., Costantino, A., Belfiore, A., Giuffrida, D., Frittitta, L., Vannelli, G. B., Brand, R., Goldfine, I. D., and Vigneri, R. Elevated insulin receptor content in human breast cancer. J. Clin. Invest., *86:* 1503–1510, 1990.

2. Papa, V., Gliozzo, B., Clark, G. M., McGuire, W. L., Moore, D., Fujita-Yamaguchi, Y., Vigneri, R., Goldfine, I. D., and Pezzino, V. Insulin-like growth factor-I receptors are overexpressed and predict a low risk in human breast cancer. Cancer Res., *53:* 3736–3740, 1993.

3. Milazzo, G., Giorgino, F., Damante, G., Sung, C., Stampfer, M. R., Vigneri, R., Goldfine, I. D., and Belfiore, A. Insulin receptor expression and function in human breast cancer cell lines. Cancer Res., *52:* 3924–3930, 1992.

4. Cullen, K. J., Yee, D., Sly, W. S., Perdue, J., Hampton, B., Lippman, M. E., and Rosen, N. Insulin-like growth factor receptor expression and function in human breast cancer. Cancer Res., *50:* 48–53, 1990.

5. Giorgino, F., Belfiore, A., Milazzo, G., Costantino, A., Maddux, B., Whittaker, J., Goldfine, I. D., and Vigneri, R. Overexpression of insulin receptors in fibroblasts and ovary cells induces a ligand-mediated transformed phenotype. Mol. Endocrinol., *5:* 452–459, 1991.

6. Kaleko, M., Rutter, W. G., and Miller, A. D. Overexpression of the human insulin-like growth factor I receptor promotes ligand-dependent neoplastic transformation. Mol. Cell. Biol., *10:* 464–473, 1990.

7. Papa, V., Costantino, A., and Belfiore, A. Insulin receptor: what role in breast cancer? Trends Endocrinol. Metab., *8:* 306–312, 1997.

8. Dickson, R. B., and Lippman, M. Growth factor in breast cancer. Endocr. Rev., *16:* 559–565, 1995.

9. Mathieu, M. C., Clark, G. M., Allred, D. C., Goldfine I. D., and Vigneri, R. Insulin receptor expression and clinical outcome in node-negative breast cancer. Proc. Assoc. Am. Physicians, *109:* 565–571, 1997.

10. Cullen, K. J., Allison, A., Martire, I., Ellis, M., and Singer, C. Insulin-like growth factor expression in breast cancer epithelium and stroma. Breast Cancer Res. Treat., *22:* 21–29, 1992.

11. Macaulay, V. M. Insulin-like growth factors and cancer. Br. J. Cancer, *65:* 311–320, 1992.

12. Moxham, C. P., Duronio, V., and Jacobs, S. Insulin-like growth factor-I receptor β-subunit heterogeneity. J. Biol. Chem., *264:* 13238–13244, 1989.

13. Soos, M. A., Whittaker, J., Lammers, R., Ullrich, A., and Siddle, K. Receptors for insulin and insulin-like growth factor-I can form hybrid dimers. Biochem. J., *270:* 383–390, 1990.

14. Kasuya, J., Paz, B. I., Maddux, B. A., Goldfine, I. D., Hefta, S. A., and Fujita-Yamaguchi, Y. Characterization of human placental insulin-like growth factor-I/insulin hybrid receptors by protein microsequencing and purification. Biochemistry, *32:* 13531–13536, 1993.

15. Soos, M. A., Field, C. E., and Siddle, K. Purified Hybrid insulin/insulin-like growth factor-I receptors bind insulin-like growth factor-I but not insulin with high affinity. Biochem. J., *290:* 419–426, 1993.

16. Soos, M. A., Siddle, K., Baron, M. D., Heward, J. M., Luzio, J. P., Bellatin, J., and Lennox, E. S. Monoclonal antibodies reacting with multiple epitopes on the human insulin receptor. Biochem. J., *235:* 199–208, 1986.

17. Forsayeth, J. R., Montemurro, A., Maddux, B. A., DePirro, R., and Goldfine, I. D. Effect of monoclonal antibodies on human insulin receptor autophosphorylation negative cooperativity and down-regulation. J. Biol. Chem., *262:* 4134–4140, 1987.

18. Roth, R. A., Cassel, D. J., Wong, K. Y., Maddux, B. A., and Goldfine, I. D. Monoclonal antibodies to the human insulin receptor block insulin binding and inhibit insulin action. Proc. Natl. Acad. Sci. USA, *79:* 7312–7316, 1982.

19. Sung, C. K., Wong, K. Y., Yip, C. C., Hawley, D. M., and Goldfine, I. D. Deletion of residues 485–599 from the human insulin receptor abolishes antireceptor antibody binding and influences tyrosine kinase activation. Mol. Endocrinol., *8:* 315–324, 1994.

20. Ganderton, R. H., Stanley, K. K., Field, C. E., Coghlan, M. P., Soos, M. A., and Siddle, K. A monoclonal anti-peptide antibody reacting with the insulin receptor β-subunit. Biochem. J., *288:* 195–205, 1992.

21. Kull, F. C., Jacobs, S., Su, Y. F., Svoboda, M. E., Van Wyk, J. J., and Cuatrecasas, P. Molecular antibody to receptor for insulin and somatomedin C. J. Biol. Chem., *258:* 6561–6566, 1983.

22. Gustafson, T. A., and Rutter, W. J. The cysteine-rich domains of the insulin-like growth factor I receptors are primary determinants of hormone binding specificity. Evidence from receptor chimeras. J. Biol. Chem., *265:* 18663–18667, 1990.

23. Soos, M. A., Field, C. E., Lammers, R., Ullrich, A., Zhang, B., Roth, R. A., Andersen, A. S., Kjeldsen, T., and Siddle, K. A panel of monoclonal antibodies for the type I insulin-like growth factor receptor. J. Biol. Chem., *267:* 12955–12963, 1992.

24. Soule, H. D., Malonei, T. M., Wolman, S. R., Peterson, W. D., Jr., Brenz, R., McGrath, C. M., Russo, J., Pauley, R. J., Jones, R. F., and Brooks, S. C. Isolation and characterization of a spontaneously immortalized human breast epithelial cell line MCF-10. Cancer Res., *50:* 6075–6086, 1990.

25. Stampfer, M. R., and Bartley, J. C. Induction of transformation and continuous cell lines from normal human mammary epithelial cells after exposure to benzo[*a*]pirene. Proc. Natl. Acad. Sci. USA, *82:* 2394–2398, 1985.

26. Stampfer, M. R. Isolation and growth of human mammary epithelial cell. J. Tissue Culture Methods, *9:* 107–115, 1985.

27. LeBon, T. R., Jacobs, S., Cuatrecasas, P., Kathuria, S., and Fujita-Yamaguchi, Y. Purification of insulin-like growth factor-I receptor human placental membranes. J. Biol. Chem., *261:* 7685–7689, 1986.

28. Baserga, R. The insulin-like growth factor-I receptor: a key to tumor growth? Cancer Res., *55:* 249–252, 1995.

29. Neuenschwander, S., Roberts, C. T., Jr., and LeRoith, D. Growth inhibition of MCF-7 breast cancer cells by stable expression of an insulin-like growth factor-I receptor antisense ribonucleic acid. Endocrinology, *136:* 4298–4303, 1995.

30. Resnicoff, M., Sell, C., Rubini, M., Coppola, D., Ambrose, D., Baserga, R., and Rubin, R. Rat glioblastoma cells expressing an antisense RNA to the insulin-like growth factor-1 (IGF-1) receptor are nontumorigenic and induce regression of wild-type tumors. Cancer Res., *54:* 2218–2222, 1994.

31. Arteaga, C. L., Kitten, L. J., Coronado, E. B., Jacobs, S., Kull, F. C., Allred, D. C., and Osborne, C. K. Blockade of the type I somatomedin receptor inhibits growth of human breast cancer cells in athymic mice. J. Clin. Invest., *84:* 1418–1423, 1989.

32. Bailyes, E. M., Nave, B. T., Soos, M. A., Orr, S. R., Hayward, A. C., and Siddle, K. Insulin receptor/IGF-I receptor hybrids are widely distributed in mammalian tissues: quantification of individual receptor species by selective immunoprecipitation and immunoblotting. Biochem J., *327:* 209–215, 1997.

33. Frattali, A. L., and Pessin, J. E. Relationship between α subunit ligand occupancy and β subunit autophosphorylation in insulin/insulin-like growth factor-I hybrid receptors. J. Biol. Chem., *268:* 7393–7400, 1993.

34. Lynn, B. S., Donna, R. R., Yasumitsu, T., Cecil, Y., and Jerrold, M. O. A functional assessment of insulin/insulin-like growth factor-I hybrid receptors. Endocrinology, *136:* 1635–1641, 1995.