IGF-I Receptor-induced Cell-Cell Adhesion of MCF-7 Breast Cancer Cells Requires the Expression of Junction Protein ZO-1*

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Hyperactivation of the insulin-like growth factor I receptor (IGF-IR) contributes to primary breast cancer development, but the role of the IGF-IR in tumor metastasis is unclear. Here we studied the effects of the IGF-IR on intercellular connections mediated by the major epithelial adhesion protein, E-cadherin (E-cad). We found that IGF-IR overexpression markedly stimulated aggregation in E-cad-positive MCF-7 breast cancer cells, but not in E-cad-negative MDA-MB-231 cells. However, when the IGF-IR and E-cad were co-expressed in MDA-MB-231 cells, cell-cell adhesion was substantially increased. The IGF-IR-dependent cell-cell adhesion of MCF-7 cells was not related to altered expression of E-cad or α-, β-, or γ-catenins but coincided with the up-regulation of another element of the E-cad complex, zonula occludens-1 (ZO-1). ZO-1 expression (mRNA and protein) was induced by IGF-I and was blocked in MCF-7 cells with a tyrosine kinase-defective IGF-IR mutant. By co-immunoprecipitation, we found that ZO-1 associates with the E-cad complex and the IGF-IR. High levels of ZO-1 coincided with an increased IGF-IR/α-catenin/ZO-1-binding and improved ZO-1/actin association, whereas down-regulation of ZO-1 by the expression of an anti-ZO-1 RNA inhibited IGF-IR-dependent cell-cell adhesion. The results suggested that one of the mechanisms by which the activated IGF-IR regulates E-cad-mediated cell-cell adhesion is overexpression of ZO-1 and the resulting stronger connections between the E-cad complex and the actin cytoskeleton. We hypothesize that in E-cad-positive cells, the IGF-IR may produce antimetastatic effects.

The insulin-like growth factor I (IGF-I)1 receptor (IGF-IR) is a ubiquitous tyrosine kinase capable of regulating different growth-related and -unrelated processes (1–3). Recent evidence indicates that the IGF-IR may be involved in breast cancer development. The IGF-IR is significantly (10–14-fold) overexpressed in estrogen receptor-positive primary breast tumors compared with normal mammary epithelium or benign tumors (1, 4). Moreover, the intrinsic ligand-independent tyrosine kinase activity of the IGF-IR has been found to be substantially up-regulated (~2–4-fold) in breast cancer cells (4). It has been suggested that the increased receptor function coupled with enhanced receptor expression amounts to a 40-fold elevation in IGF-IR activity in estrogen receptor-positive breast tumors (4). Recent clinical and experimental data indicate that up-regulation of IGF-IR signaling in estrogen receptor-positive breast cancer cells is associated with autonomous cell proliferation, estrogen-independence, and increased resistance to various antitumor treatments (1). Consequently, it is believed that hyperactivation of the IGF-IR may induce and sustain the growth of primary breast tumors (1).

The role of the IGF-IR in breast cancer metastasis, however, is unclear. The experimental data suggest that the IGF-IR has a function in cell spreading by effectively stimulating the motility of different metastatic breast cancer cell lines lacking the expression of a major adhesion protein, E-cadherin (E-cad) (1, 5, 6). On the other hand, we and others have shown that in more differentiated E-cad-positive cells, IGF-I treatment or IGF-IR overexpression up-regulates cell-cell adhesion, which correlates with increased cell survival in three-dimensional culture and with reduced cell migration in vitro and in organ culture (1, 7–10).

The mechanism of IGF-I-dependent intercellular adhesion and the clinical consequences of this phenomenon have not been fully elucidated. Previously, we demonstrated that in MCF-7 human breast cancer cells, the IGF-IR co-localizes and co-precipitates with the E-cad complex and that IGF-induced aggregation is blocked with an anti-E-cad antibody (7). In this study we assessed the effects of the IGF-IR on the elements of the E-cad adhesion complex, i.e. E-cad; β-, γ-, and α-catenins; and α-catenin-associated proteins (see Fig. 6). The initial results prompted us to focus on an α-catenin-binding element, the junction protein zonula occludens-1 (ZO-1).

ZO-1 is a ~220-kDa scaffolding protein containing various domains (an SH3 domain, three PDZ domains, a proline-rich region, and a guanylate kinase domain) that allow its interaction with specialized sites of plasma membrane as well as with other proteins (11, 12). ZO-1 is a characteristic element of tight junctions, but recently its presence has also been demonstrated in E-cad adherens junctions (13–15). The role of ZO-1 in adherens junctions is yet unclear, but it is assumed that it may functionally link E-cad with the actin cytoskeleton because it associates with α-catenin and actin through its N and C termini, respectively (Ref. 13, see Fig. 6). In addition, as a member of the membrane-associated guanylate kinase homologue (MAGUK) family of putative signaling proteins, ZO-1 may be involved in signal transduction. Indeed, ZO-1 has been found to

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1 The abbreviations used are: IGF-I, insulin-like growth factor I; IGF-IR, IGF-I receptor; E-cad, E-cadherin; ZO-1, zonula occludens-1 junction protein; Y3F, Tyr-1131, Tyr-1135, and Tyr-1136 replaced with Phe; WB, Western blotting or Western blotted; pAb, polyclonal antibody; mAb, monoclonal antibody; ERK1, extracellular signal-related kinase 1; IRS-1, insulin receptor substrate 1; MAPK, mitogen-activated protein kinase.

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Effects of IGF-IR and ZO-1 expression on cell aggregation in E-cad-positive and -negative breast cancer cells

The stable cell lines (MCF-7, MCF-7/IGF-IR, MCF-7/IGF-IR-anti-ZO-1, MCF-7/Y3F, MDA-MB-231, and MDA-MB-231/IGF-IR) and transiently transfected populations (MDA-MB-231/E-cad, MDA-MB-231/vector, MDA-MB-231/IGF-IR/e-cad, and MDA-MB-231/IGF-IR/vector) were cultured as three-dimensional spheroids in normal growth medium. The number of spheroids of different sizes was established as described under “Materials and Methods.”

The stable cell lines (MCF-7, MCF-7/IGF-IR, MCF-7/IGF-IR/anti-ZO-1, MCF-7/Y3F, MDA-MB-231, and MDA-MB-231/IGF-IR) were transiently transfected with the IGF-IR/Y3F vector (see below). MCF-7/IGF-IR/anti-ZO-1 and MCF-7/IGF-IR/sense ZO-1 cells were derived from MCF-7/IGF-IR clone 15 by stable transfection with the antisense and sense ZO-1 vectors, respectively and subsequent selection in 500 μg/ml hygromycin B. MDA-MB-231 is a metastatic breast cancer cell line lacking E-cad and expressing ~7 × 10^5 IGF-IR receptors/cell (24). MDA-MB-231/IGF-IR clone 31 was derived from MDA-MB-231 cells by stable transfection with the pcDNA3/IGF-IR plasmid. MDA-MB-231/IGF-IR cells express ~250,000 IGF-IR/cell.

All cell lines were grown in Dulbecco’s modified Eagle’s medium/F12 (1:1) containing 5% calf serum. MCF-7- and MDA-MB-231-derived clones transfected with the wild-type or mutant IGF-IR were maintained in growth medium with 100 μg/ml G418. MCF-7/IGF-IR/anti-ZO-1 and MCF-7/IGF-IR/sense ZO-1 cells were cultured in growth medium with 50 μg/ml hygromycin B. In the experiments requiring serum-free conditions, the cells were cultured in phenol red-free Dulbecco’s modified Eagle’s medium containing 0.5 mg/ml bovine serum albumin, 1 μM FeSO4, and 2 mM L-glutamine (referred to as SFM). Transient Transfection

MDA-MB-231 and MDA-MB-231/IGF-IR cells were transiently transfected using LipofectAMINE 2000 (Life Technologies, Inc.) (reagent/DNA ratio, 5 μl/1 μg). The transfection was carried out in growth medium for 24 h, and then the cells were lysed and processed for E-cad Western blotting (WB). To evaluate the extent of cell-cell adhesion in the transfected MDA-MB-231 and MDA-MB-231/IGF-IR cells, the cells were trypsinized upon transfection, counted, and placed in three-dimensional suspension culture as described below. MCF-7 cells were transfected for 6 h in growth medium using FuGene 6 (Roche Molecular Biochemicals) (reagent/DNA ratio, 3 μl/1 μg). To study IGF-1 signaling, the transfected MCF-7 cells were shifted to SFM for 36 h and stimulated with IGF-1 for 15 min. The efficiency of transfection (transfected cells/total cell number) was at least 70% for all cell types and was estimated by scoring fluorescent cells in cultures transfected with the plasmid pCMS (encoding green fluorescent protein) (Invitrogen).

Three-dimensional Spheroid Culture

The cells were grown to 70–80% confluence, trypsinized, and plated in single-cell suspension in 2%-agar-coated plates containing either normal growth medium or SFM. 2 × 10^6 cells were plated per 100 mm culture dish. To generate three-dimensional spheroids, the plates were rotated for 1 h at 37°C. The spheroids started to assemble at ~1 h after plating and were completely organized after 3–4 h of culture in suspension. The three-dimensional cultures were photographed using a phase-contrast microscope (Nikon or Olympus). The extent of aggregation was scored by measuring the spheroids with an ocular micrometer. For each cell type, the spheroids between 25 and 50, 50 and 100, and >100 μm (in the smallest cross-section) were counted in 10 different fields under ×10 magnification.

IGF Stimulation

70% confluent cell cultures were synchronized in SFM for 36 h and then stimulated with 20 ng/ml IGF-I for 0–72 h. Immunoprecipitation and Western Blotting

The expression of different elements of the adhesion complex was assessed in 500 μg of protein lysate by immunoprecipitation and WB

### Table I

| Cells | Spheroids |
|-------|-----------|
|       | 25 ± 50 μm | 50 ± 100 μm | >100 μm |
| MCF-7 | 21.0 ± 1.9 | 96.0 ± 6.7 | 2.7 ± 0.9 |
| MCF-7/IGF-IR | 1.7 ± 0.7 | 22.3 ± 1.4 | 86.5 ± 3.9 |
| MCF-7/IGF-IR/anti-ZO-1 | 75.0 ± 3.5 | 40.7 ± 2.1 | 0.0 ± 0.0 |
| MCF-7/Y3F | 45.0 ± 2.9 | 39.8 ± 4.6 | 0.0 ± 0.0 |
| MDA-MB-231 | 7.0 ± 0.6 | 0.5 ± 0.2 | 0.0 ± 0.0 |
| MDA-MB-231/IGF-IR | 12.5 ± 0.8 | 0.5 ± 0.1 | 0.0 ± 0.0 |
| MDA-MB-231/E-cad | 39.8 ± 3.6 | 15.2 ± 1.1 | 0.0 ± 0.0 |
| MDA-MB-231/vector | 10.0 ± 1.4 | 0.6 ± 0.4 | 0.0 ± 0.0 |
| MDA-MB-231/IGF-IR/E-cad | 27.0 ± 2.2 | 68.3 ± 7.2 | 8.0 ± 1.5 |
| MDA-MB-231/IGF-IR/vector | 18.6 ± 2.2 | 0.9 ± 0.3 | 0.0 ± 0.0 |

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with appropriate antibodies. The expression of ERK1/ERK2 was tested in 50 μg of total cell lysate. The cell lysis buffer contained 50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM CaCl₂, 100 mM NaF, 0.2 mM Na₃VO₄, 1% phenylmethylsulfonyl fluoride, and 1% aprotinin as described before (25). The following antibodies were used: anti-ZO-1 polyclonal antibody (pAb) (Zymed Laboratories Inc.) for ZO-1 immunoprecipitation (5 μg/ml) and WB (2 μg/ml); anti-ERK monoclonal antibody (mAb), clone 3D-12 (Zymed Laboratories) for ZO-1 immunoprecipitation (4 μg/ml) and WB (0.5 μg/ml); anti-α-catenin pAb (Sigma or Zymed Laboratories Inc.) for α-catenin immunoprecipitation (4 μg/ml) and WB (anti-serum dilution 1:4000); anti-phospho-MAPK mAb (Transduction Laboratories) for β-catenin immunoprecipitation (4 μg/ml) and WB (0.5 μg/ml); anti-γ-catenin pAb (Sigma) for γ-catenin WB (1 μg/ml); anti-actin mAb clone AC-40 (Sigma) for actin WB (0.4 μg/ml); anti-IGF-IR mAb, clone aIR-3 (Calbiochem) for IGF-IR immunoprecipitation (3 μg/ml), and anti-IGF-IR pAb C-20 (Santa Cruz Biotechnology) for IGF-IR WB (0.2 μg/ml); anti-p85 pAb (Upstate Biotechnology, Inc.) for the p85 subunit of the phosphatidylinositol 3-kinase WB (0.25 μg/ml); anti-phospho-MAPK mAb (New England Biolabs, Inc.) for active ERK1/ERK2 WB (0.5 μg/ml); and anti-MAPK pAb (New England Biolabs) for total ERK1/ERK2 WB (1 μg/ml). Tyrosine phosphorylation of immunoprecipitated proteins was measured by WB with anti-phosphotyrosine mAb (Transduction Laboratories) (0.03 μg/ml). Western blots were developed using an ECL chemiluminescence kit (Amersham Pharmacia Biotech). The intensity of bands representing relevant proteins was measured by laser densitometry scanning.

RESULTS

IGF-IR Overexpression Stimulates Cell-Cell Adhesion through an E-cad-dependent Mechanism—First, we demonstrated that under three-dimensional culture conditions, overexpression of the IGF-IR stimulated cell-cell adhesion in E-cad-positive MCF-7 breast cancer cells but not in E-cad-negative MDA-MB-231 cells (Fig. 1, A and B) (Table I). However, co-expression of the IGF-IR and E-cad resulted in robust cell-cell adhesion of MDA-MB-231 cells, whereas the expression of E-cad alone was less efficient in inducing intercellular contacts (Fig. 1, C and D) (Table I). These results, together with our previous data showing that IGF-IR-mediated aggregation in MCF-7 cells is blocked with an anti-E-cad antibody (7), indicated that IGF-IR adhesion signals are transmitted through the E-cad complex.

IGF-IR Overexpression Up-regulates ZO-1—We tested whether high levels of the IGF-IR affect the expression of the proteins within the E-cad complex and found that in MCF-7 and MCF-7/IGF-IR cells cultured as three-dimensional spheroids, the levels of E-cad and α-, β-, and γ-catenin were similar. However, the abundance of ZO-1 was significantly increased in MCF-7/IGF-IR cells (Fig. 2). The tyrosine phosphorylation of all these adhesion proteins was undetectable in spheroids and was not influenced by IGF-IR overexpression (Fig. 5 and data not shown).

To investigate whether the increased expression of ZO-1 in MCF-7/IGF-IR cells depends on IGF-IR tyrosine kinase activity, we generated by stable or transient transfection MCF-7/IGF-IR/Y3F cells expressing high levels of a kinase-defective IGF-IR mutant (IGF-IR/Y3F). The overexpression of the IGF-IR/Y3F mutant resulted in impaired IGF-I response, which was reflected by markedly reduced IGF-IR and IRS-1 tyrosine phosphorylation, decreased IRS-1/p85 binding, and diminished ERK1/ERK2 stimulation (Fig. 3). The basal expression of ZO-1 in MCF-7/IGF-IR/Y3F cells was significantly reduced compared with that in MCF-7/IGF-IR cells, indicating that tyrosine kinase activity of the IGF-IR is required for the up-regulation of ZO-1 (Fig. 3). Interestingly, the inhibition of IGF-IR response did not affect E-cad expression, suggesting a selective action of the IGF-IR toward ZO-1 (Fig. 3). The blockade of the IGF-IR signal in MCF-7/IGF-IR/Y3F cells coincided with reduced cell-cell adhesion (Table 1).

ZO-1 mRNA and Protein Expression Is Regulated by IGF-IR—To establish whether the activation of the IGF-IR by IGF produces a similar effect on ZO-1 as that seen with IGF-IR overexpression, we studied ZO-1 mRNA and protein in MCF-7 and MCF-7/IGF-IR cells treated with 20 ng/ml IGF-I for 1–72 h (Fig. 4). In MCF-7 cells cultured in SFM, the basal levels of ZO-1 mRNA were low and were markedly increased between 4 and 36 h of IGF-I treatment (Fig. 4A). In contrast, the abundance of ZO-1 mRNA was always elevated in MCF-7/IGF-IR cells and was only moderately improved by IGF-I (4–72 h) (Fig.
IGF-IR expresses 12, 17, and 15, respectively (7).

Anti-IGF-IR or anti-E-cad antibodies revealed reduced expression of ZO-1, which reflected the expression of ZO-1 mRNA (Fig. 4A). ZO-1 protein levels in IGF-I-treated cells generally remained unchanged. Similarly, high levels of the IGF-IR did not affect the expression and increased expression of E-cad and ZO-1 have consequences for the association of the proteins within the E-cad complex. Co-immunoprecipitation experiments demonstrated that IGF-IR overexpression resulted in an increased abundance of IGF-IR/E-cad and E-cad/α-catenin (a ZO-1-associated protein) to the IGF-IR or ZO-1 but not to E-cad was greater in MCF-7/IGF-IR cells than in MCF-7 cells (Fig. 5A). The presence of α-catenin in IGF-IR immunoprecipitates was confirmed with cell lysates in which α-catenin was first removed with a specific antibody. As expected, immunoprecipitation of such depleted lysates with either anti-IGF-IR or anti-E-cad antibodies revealed reduced α-catenin/E-cad and α-catenin/IGF-IR associations (Fig. 5B). Further experiments with α-catenin immunoprecipitates indicated increased abundance of α-catenin-actin and α-catenin-ZO-1 complexes in MCF-7/IGF-IR cells (Fig. 5C). A hypothetical model of possible interactions between adhesion proteins and the IGF-IR is shown in Fig. 6.

**Fig. 2.** Expression of adhesion proteins in MCF-7 and MCF-7/IGF-IR cells. The expression of adhesion proteins and the IGF-IR was studied in 50 µg of protein lysates obtained from cells cultured as three-dimensional spheroids in normal growth medium. MCF-7/IGF-IR cells are pooled MCF-7/IGF-IR clones 12, 15, and 17 (see “Materials and Methods”). A, the levels of the IGF-IR, E-cad; α-, β-, γ-catenin (cat); and ZO-1 detected by WB using specific antibodies (see “Materials and Methods”). B, the expression of ZO-1 in MCF-7 cells and in MCF-7/IGF-IR clones (cl.) 12, 17, and 15, expressing ~5 × 10⁵, 1 × 10⁶, and 3 × 10⁶ IGF-IR/cell, respectively (7).

**Fig. 3.** ZO-1 expression is inhibited in MCF-7/IGF-IR/Y3F cells. MCF-7/IGF-IR cells expressing the wild-type IGF-IR (WT) and MCF-7/IGF-IR/Y3F cells stably transfected with a dominant-negative kinase-defective IGF-IR mutant (Y3F) were synchronized in SFM (time 0) and then stimulated with 20 ng/ml IGF-I for different times (1–72 h). The expression of 7.8-kilobase ZO-1 mRNA in MCF-7 and MCF-7/IGF-IR cells was studied by Northern blotting in 20 µg of total RNA using a [32P]dCTP-labeled ZO-1 probe (described under “Materials and Methods”). The expression and tyrosine phosphorylation (PY) of the IGF-IR and IRS-1 in the cells were detected by immunoprecipitation and WB in 500 µg of protein lysates. The binding of the p85 subunit of the phosphatidylinositol 3-kinase to IRS-1 (IRS-1/p85) was studied by WB in IRS-1 immunoprecipitates. The expression of active ERK1/ERK2 (p-ERK1/2), total ERK1/2, ZO-1, and E-cad was evaluated by WB in 50 µg of total protein lysates. The specific antibodies used are listed under “Materials and Methods.” Similar results were obtained with MCF-7 cells transiently transfected with the IGF-IR/Y3F expression vector.

**Fig. 4.** ZO-1 mRNA and protein are regulated by IGF-I. A, MCF-7 cells and MCF-7/IGF-IR were synchronized in SFM (time 0) and then stimulated with 20 ng/ml IGF-I for different times (1–72 h). The expression of 7.8-kilobase ZO-1 mRNA in MCF-7 and MCF-7/IGF-IR cells was studied by Northern blotting in 20 µg of total RNA using a [32P]dCTP-labeled ZO-1 probe (described under “Materials and Methods”). B, 28 and 18 S rRNA are shown as a control of RNA loading. The expression of active ERK1/ERK2 (p-ERK1/2), total ERK1/2, ZO-1, and E-cad was evaluated by WB in 50 µg of total protein lysates. The specific antibodies used are listed under “Materials and Methods.” Similar results were obtained with MCF-7 cells transiently transfected with the IGF-IR/Y3F expression vector.
Down-regulation of ZO-1 Results in Decreased Cell-Cell Adhesion in MCF-7/IGF-IR Cells—Because the results suggested that ZO-1 may be an important intermediate in IGF-IR-stimulated cell-cell adhesion, we set out to confirm this notion using MCF-7/IGF-IR cells in which ZO-1 levels were down-regulated by the expression of an anti-ZO-1 RNA (MCF-7/IGF-IR/anti-ZO-1 cells) (Fig. 7). The clones with the best ZO-1 reduction and an intact E-cad and IGF-IR expression were analyzed in three-dimensional culture. The cell-cell adhesion of MCF-7/IGF-IR/anti-ZO-1 cells was greatly inhibited compared with that in the parental MCF-7/IGF-IR cells (Fig. 7 and Table 1). The expression of the anti-ZO-1 plasmid in the parental MCF-7 cells was toxic, and no viable clones were obtained. The transfection of the sense-ZO-1 vector had no effect on cell-cell adhesion (data not shown).

DISCUSSION

Cell-cell adhesion is a known factor modulating the motility of tumor cells and consequently impacting tumor metastasis (26). The regulation of this process by exogenous growth factors is still not well understood. In E-cad-positive breast cancer cells, the overexpression or activation of the IGF-IR has been shown to stimulate cell-cell adhesion and reduce cell spreading in vitro or in organ culture (7–10). The IGF-IR has also been found co-localized and co-precipitated with the E-cad adhesion complex (7, 8). The mechanism of IGF-IR-stimulated E-cad-dependent cell-cell adhesion is unknown and has been investigated in this work. We discovered the following observations. 1) IGF-IR overexpression increased aggregation in E-cad-positive cells but not in E-cad-negative cells. 2) High expression of both IGF-IR and E-cad markedly improved cell aggregation in E-cad-negative cells. 3) IGF-IR-dependent cell-cell adhesion in E-cad-positive cells did not affect the expression of E-cad or α-, β-, or γ-catenin but coincided with up-regulation of ZO-1. 4) ZO-1 expression was induced by IGF-I and required IGF-IR tyrosine kinase activity, and 5) high levels of ZO-1 coincided with an increased IGF-IR/α-catenin/ZO-1 binding and improved ZO-1/actin association, whereas down-regulation of ZO-1 by the expression of an anti-ZO-1 RNA inhibited IGF-IR-dependent cell-cell adhesion. We hypothesize that the mechanism or one of the mechanisms by which the activated IGF-IR stimulates cell-cell adhesion is overexpression of ZO-1 and the resultant stronger connections between the E-cad complex and the actin cytoskeleton.

Very little is known about the regulation of ZO-1 by growth factors in breast cancer cells compared with that seen in A. C, 500 μg of protein lysates were precipitated with anti-α-catenin antibody and probed by WB for α-catenin, actin, and ZO-1. The blots presented in A, B, and C were identically developed with film exposure time 10 s.
IGF-IR Up-regulates Adhesion through ZO-1

Materials and Methods.

The levels of ZO-1, IGF-IR, and E-cad in the parental MCF-7/IGF-IR cells (1, 28, 29) were studied by WB in 50 μg of protein lysate. The aggregation of cells was studied in three-dimensional culture as described under “Materials and Methods.” The bar in B represents 50 μm.

Several growth factors (e.g. epidermal growth factor and vascular endothelial growth factor) have been demonstrated to increase tyrosine phosphorylation of ZO-1 in different cellular model systems (18, 19). Hyperphosphorylation of ZO-1 usually coincides with its departure from tight junctions into the cytoplasm and with increased permeability (18, 19). In addition, v-Src-increased ZO-1 tyrosine phosphorylation has been linked to decreased cell-cell adhesion (27). IGF-IR, on the other hand, has been shown to stabilize ZO-1 in tight junctions and to preserve the epithelial barrier in embryonic kidney cells and in pig thyrocytes (28, 29). However, the effects of IGF-IR on ZO-1 expression and function in breast cancer cells have never been explored. Our findings provide the first evidence that the activation of the IGF-IR up-regulates ZO-1 mRNA and protein levels without affecting ZO-1 tyrosine phosphorylation. Consistent with the results obtained in other models, we noted increased adhesion in cells overexpressing ZO-1 and reduced aggregation in cells with down-regulated ZO-1 levels.

IGF-IR tyrosine phosphorylation was required for the stimulation of ZO-1 expression, inasmuch as the basal levels of ZO-1 were not increased in MCF-7/IGF-IR/Y3F cells expressing a dominant-negative, kinase-defective mutant of the IGF-IR. However, the putative IGF-IR signaling pathways leading to ZO-1 expression have yet to be characterized. Our preliminary data with MCF-7/IRS-1 cells, in which the major IGF-IR/IRS-1/phosphatidylinositol 3-kinase growth/survival pathway is hyperactivated (1), suggested that this pathway is not involved in ZO-1 regulation (data not shown).

The clinical implications of IGF-induced and ZO-1-mediated cell-cell adhesion on tumor development and progression are unknown. Until now, the data from our and other laboratories suggest that in E-cad-positive breast cancer cells IGF-IR improves cell-cell adhesion and cell survival in three-dimensional culture but at the same time reduces cell spreading (1). Thus, one consequence of IGF-IR overexpression in breast cancer could be increased growth and survival of the primary tumor but reduced cell metastasis. This hypothesis is consistent with the observation that the IGF-IR is a good prognostic indicator for breast cancer, as tumors with good prognosis express much higher levels of the IGF-IR than tumors with bad prognosis (1, 30, 31). Notably, an independent study has shown that in breast tumors, E-cad and ZO-1 are co-expressed and are markers of a more differentiated phenotype (17). A formal analysis of the correlations between ZO-1 and the IGF-IR is underway in our laboratory and should help in clarifying the role of the IGF-IR in breast cancer progression.

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