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Expression of the insulin-like growth factor 1 receptor (IGF-1R) in breast cancer cells: evidence for a regulatory role of dolichyl phosphate in the transition from an intracellular to an extracellular IGF-1 pathway

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In this study we provide evidence that the low expression of IGF-1R at the cell surface of estrogen-independent breast cancer cells is due to a low rate of *de novo* synthesis of dolichyl phosphate. The analyses were performed on the estrogen receptor-negative breast cancer cell line MDA231 and, in comparison, the melanoma cell line SK-MEL-2, which expresses a high number of plasma membrane-bound IGF-1R. Whereas the MDA231 cells had little or no surface expression of IGF-1R, they expressed functional (i.e., ligand-binding) intracellular receptors. By measuring the incorporation of [^{3}H]mevalonate into dolichyl phosphate, we could demonstrate that the rate of dolichyl phosphate synthesis was considerably lower in MDA231 cells than in SK-MEL-2 cells. Furthermore, N-linked glycosylation of the α-subunit of IGF-1R was 8-fold higher in the melanoma cells. Following addition of dolichyl phosphate to MDA231 cells, N-linked glycosylation of IGF-1R was drastically increased, which in turn was correlated to a substantial translocation of IGF-1R to the plasma membrane, as assayed by IGF-1 binding analysis and by Western blotting of plasma membrane proteins. The dolichyl phosphate–stimulated receptors were proven to be biochemically active since they exhibited autophosphorylation. Under normal conditions MDA231 cells, expressing very few IGF-1R at the cell surface, were not growth-arrested by an antibody (αIR-3) blocking the binding of IGF-1 to IGF-1R. However, after treatment with dolichyl phosphate, leading to a high cell surface expression of IGF-1R, αIR-3 efficiently blocked MDA231 cell growth. Taken together with the fact that the breast cancer cells produce IGF-1 and exhibit intracellular binding, our data suggest that the level of *de novo*–synthesized dolichyl phosphate may be critical for whether the cells will use an intracellular or an extracellular autocrine IGF-1 pathway.

Key words: tunicamycin/N-linked glycosylation/IGF-1 receptor/breast cancer cells

Introduction

IGF-1R is a heterotetrameric plasma membrane glycoprotein composed of two α-subunits (130 kDa each) and two β-subunits (90 kDa each) linked by disulfide bonds (Massagué and Czech, 1992). IGF-1 is necessary in many tumor cell types for the establishment and maintenance of the transformed phenotype and for tumorigenesis (Kalebic *et al*., 1994; Resnicoff *et al*., 1994; Shapiro *et al*., 1994). IGF-1R has been shown to protect cells from apoptosis (Baserga, 1995; Resnicoff *et al*., 1995), and it has also been observed that inhibition of tumorigenesis, induced by an impaired function of IGF-1 receptors, is correlated to the ability of the IGF-1 receptor to protect transformed cells from apoptosis (Harrington *et al*., 1994; Prager *et al*., 1994; Sell *et al*., 1995).

In a previous study we have demonstrated that inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase leads to an impaired translocation of IGF-1R proteins to the cell surface in melanoma cells (Carlberg *et al*., 1996). The downregulation of IGF-1R was correlated to a decrease in *de novo* synthesis of dolichyl phosphate and in N-linked glycosylation of the receptor proteins, as well as to an inhibition of cell growth (Carlberg *et al*., 1996). These data suggested that dolichyl phosphate–dependent glycosylation of IGF-1R is involved in mevalonate-regulated cell growth. Similar results have also been shown in normal human fibroblasts (Carlberg and Larsson, 1996).

In another study we have shown that a prolonged inhibition of N-linked glycosylation, using tunicamycin (TM), in melanoma cells induces apoptosis through downregulation of the IGF-1 receptors at the cell surface (Dricu *et al*., 1997a). In this way the effect of TM simulates the effect of growth factor depletion, which in fact leads to apoptosis in tumor-transformed cells (Evan *et al*., 1992). Other malignant cell types, including breast cancer cells (i.e., MDA231 cells), also underwent cell death after treatment with TM, however not through its inhibitory effect on cell surface expression of IGF-1R (Dricu *et al*., 1997a).

In the present study we aimed to investigate the role of dolichyl phosphate–dependent glycosylation in regulation of IGF-1R expression in breast cancer cells. Estrogen receptor-positive breast cancer cells have been shown to express a high number of IGF-1R at the cell surface whereas estrogen receptor-negative ones express few receptors (Peyrat and Bonneterre, 1992). Furthermore, there seems to be a positive correlation between the number of IGF-1R and favorable prognosis in breast cancer (Peyrat and Bonneterre, 1992). We could confirm that the commonly used estrogen-independent breast cancer cell line MDA231, which represents a highly malignant phenotype, expresses few or no IGF-1R at the cell surface. However, they expressed intracellular receptors that bind IGF-1. Our data suggest that IGF-1R were retained intracellularly because they were not fully glycosylated, which in turn was found to correlate to a comparatively low rate of dolichyl phosphate synthesis in these cells.

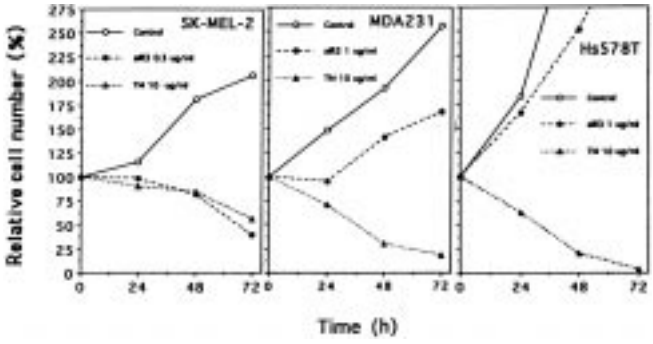

Fig. 1. Effect of TM and αIR-3 on growth and survival of melanoma and breast cancer cells. Exponentially growing SK-MEL-2 (**A**), MDA231 (**B**), and Hs578T (**C**) cells were subjected to continuous treatment with TM (10 μg/ml) or αIR-3 (0.3 μg/ml in SK-MEL-2, and 1.0 μg/ml in MDA231 and Hs578T) for 72 h. Cells were counted at every 24 h. The mean values of two determinations are shown. All standard deviations were less than 10% of the means. The effect of TM at 48 h was proven to be statistically significant ($p < 0.05$ to $p < 0.001$) in all three cell lines.

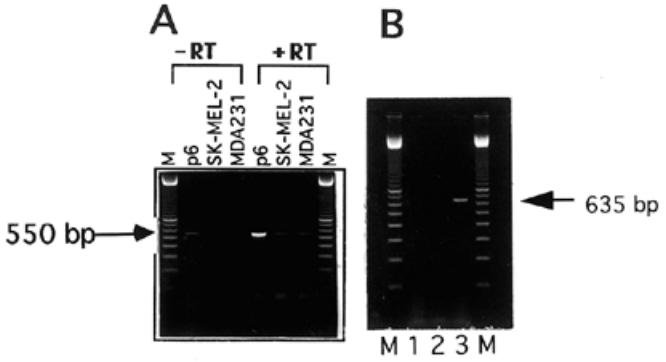

Fig. 2. Expression of IGF-1R and IGF-1 genes. (**A**) RNA was isolated from p6, SK-MEL-2, and MDA231 cells, whereupon RT-PCR was performed using cDNA primers for IGF-1R mRNA (see *Materials and methods*). The size of the positive PCR product for IGF-1R is 550 bp. +RT and -RT indicate whether reverse transcription was run or not run before the PCR step, respectively. M indicates molecular marker. (**B**) RT-PCR using cDNA primers for IGF-1 transcripts was run on RNA isolated from MDA231 cells. The size of the PCR product for IGF-1 is 635 bp. Lane 1, PCR on MDA231 mRNA without RT; lane 2, negative PCR control; lane 3, RT-PCR on MDA231 mRNA.

Results

We aimed to investigate the role of dolichyl phosphate–dependent glycosylation in regulation of IGF-1R expression in breast cancer cells. Hereby, most experiments were performed on MDA231 cells. We compared this cell line with SK-MEL-2 cells, and in some experiments with Hs578T and p6 cells. SK-Mel-2 cells have recently been extensively characterized with regard to the regulatory role of N-linked glycosylation in expression of IGF-1R at the cell surface (Carlberg *et al*., 1996; Dricu *et al*., 1997b). Hs578T functions as a negative control since this cell line obviously does not express an intact IGF-1R (Peyrat and Bonneterre, 1992). p6 cells overexpress IGF-1R (Pietrzkowski *et al*., 1992). In this study the cells were maintained in medium containing 10% serum during the whole experiments.

Inhibition of N-linked glycosylation was induced by tunicamycin (TM) (10 μg/ml), and the blockade of the binding sites of IGF-1R by the antibody αIR-3. The effect of TM and αIR-3 on cell growth and survival was analyzed on a human melanoma cell line (SK-MEL-2) and two estrogen receptor-negative human breast cancer cell lines (MDA231 and Hs578T). The proliferation of SK-MEL-2 ceased immediately after addition of TM and αIR-3 (0.3 μg/ml) (Figure 1A). The number of cells decreased gradually, and after 72 h only 40–60% of the cells were alive. From a kinetic point of view the response to TM and αIR-3 was similar (Figure 1A). In MDA231 (Figure 1B) and Hs578T (Figure 1C) TM blocked the proliferation and decreased cell viability drastically. In contrast, treatment with αIR-3, although added at a concentration of 1.0 μg/ml, only had a slight inhibitory effect on proliferation of the breast cancer cells (Figure 1B,C). As a control an antibody blocking the ligand-binding site of the epidermal growth factor receptor was also tested. However, it had no significant inhibitory effect on either cell growth or survival in these three cell lines (data not shown).

Using RT-PCR it was confirmed that the IGF-1R gene is expressed in p6 (3T3-cells containing a plasmid with a constitutively expressed human IGF-1R gene), SK-MEL-2 and MDA231 cells (Figure 2A). The positive signal (550 bp) in the negative RT control (i.e., PCR without previous reversed transcription) of p6 cells is explained by that this cell line carries a plasmid containing cDNA for human IGF-1R. A positive PCR band (635 bp) appeared after RT-PCR of MDA231 mRNA using IGF-1 primers (Figure 2B). This implies that the IGF-1 gene is expressed in this cell line, which in turn suggests that IGF-1 is produced. IGF-1 transcripts were also detected in SK-MEL-2 but not in p6 cells (data not shown).

In Figure 3A it is demonstrated that, in contrast to SK-MEL-2, there was hardly any surface binding of IGF-1 in the two breast cancer cell lines. These data suggest that there are very few functional IGF-1Rs in the plasma membrane of MDA231 and Hs578T cells. Regarding the Hs578T cell line, this result probably is explained by the fact that it expresses atypical receptors (Peyrat and Bonneterre, 1992). In the following experiments we only used SK-MEL-2 and MDA231 cells. Results obtained by cross-linking of ^{125}I-IGF-1 to IGF-1R (Figure 3B) and Scatchard plot analysis (Figure 3C) confirm that the breast cancer cells contain few or no binding sites at the cell surface.

By performing Western blotting using an antibody against the α-subunit of IGF-1R, the amounts of plasma membrane-bound and intracellular receptors in SK-MEL-2 and MDA 231 cells were compared (Figure 4A). As distinguished from SK-MEL-2 there was no detectable 130 kDa IGF-1R α-subunit in the plasma membrane of the MDA 231 cells. However, as also seen in the melanoma cells, IGF-1R protein was detected in the intracellular compartment (i.e., ER) of MDA231. In both cell lines the intracellular IGF-1R exhibited a molecular weight of ~120 kDa instead of 130 kDa (Figure 4A). A probable explanation for this is that the intracellular receptor proteins are glycosylated to a lesser extent than the plasma membrane-bound ones. In Figure 4B it is demonstrated that solubilized IGF-1Rs from MDA231 cells express a significant binding activity comparable to that in SK-MEL-2. This suggests that the breast cancer cells express functional *intracellular* IGF-1R.

The rate of N-linked glycosylation of the α-subunit of IGF-1R in SK-MEL-2 and MDA231 cells was now compared. This was performed by measuring the incorporation of [^{3}H]glucosamine into IGF-1R, which after labeling of the cells with [^{3}H]glucosa-

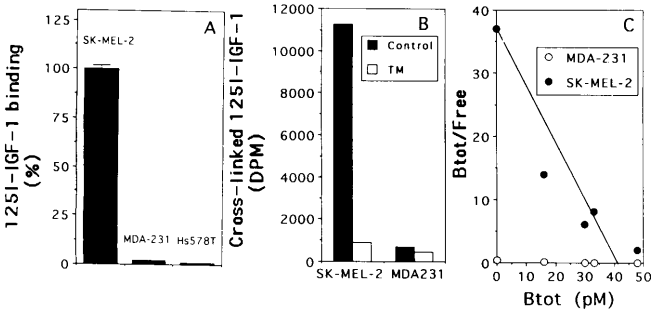

Fig. 3. Comparison of IGF-1 binding in melanoma cells and breast cancer cells. (**A**) SK-MEL-2, MDA231 and Hs578T were incubated in regular medium for 24 h whereupon they were subjected to assay of ^{125}I-IGF-1 binding. The values represent % binding activity related to SK-MEL-2 cells (30,000 DPM/mg protein). Mean values and SD values are shown. (**B**) SK-MEL-2 and MDA231 cells were changed to new medium with or without TM (10 μg/ml). After 24 h the cells were labeled with ^{125}I-IGF-1 after which cross-linking was performed. Radioactivity in isolated proteins from each dish was then counted in a scintillation counter. (**C**) SK-MEL 2 and MDA 231 cells growing in regular medium were incubated with ^{125}I-IGF-1 and different concentrations of unlabeled ligand (10–1000 ng/ml), after which Scatchard plot analysis was performed. The values are the means for duplicate determinations.

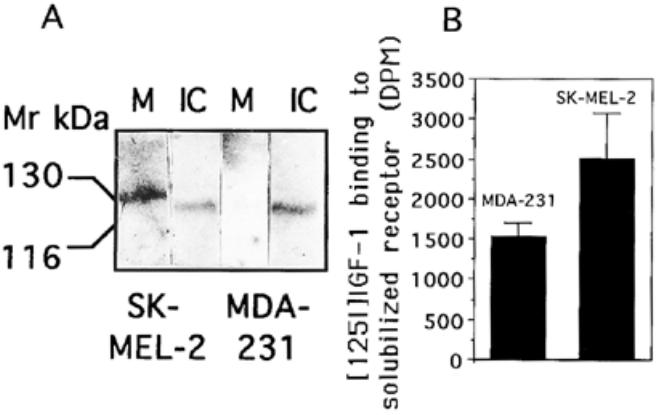

Fig. 4. The expression of IGF-1R proteins in melanoma and breast cancer cells. (**A**) SK-MEL-2 and MDA231 cells were harvested for isolation of plasma membrane-bound proteins and intracellular proteins, after which Western blotting using an antibody (N-20) against the α-subunit of IGF-1R was run. Molecular weights are indicated. M, plasma membrane; IC, intracellular compartment (i.e., cytosol and organelles). (**B**) SK-MEL-2 and MDA231 cells were harvested for analysis of ^{125}I-IGF-1 binding to solubilized IGF-1R. The specific ^{125}I-IGF-1 binding was obtained by reducing the nonspecific binding, which was determined after coincubation with unlabeled IGF-1 (1000 ng/ml).

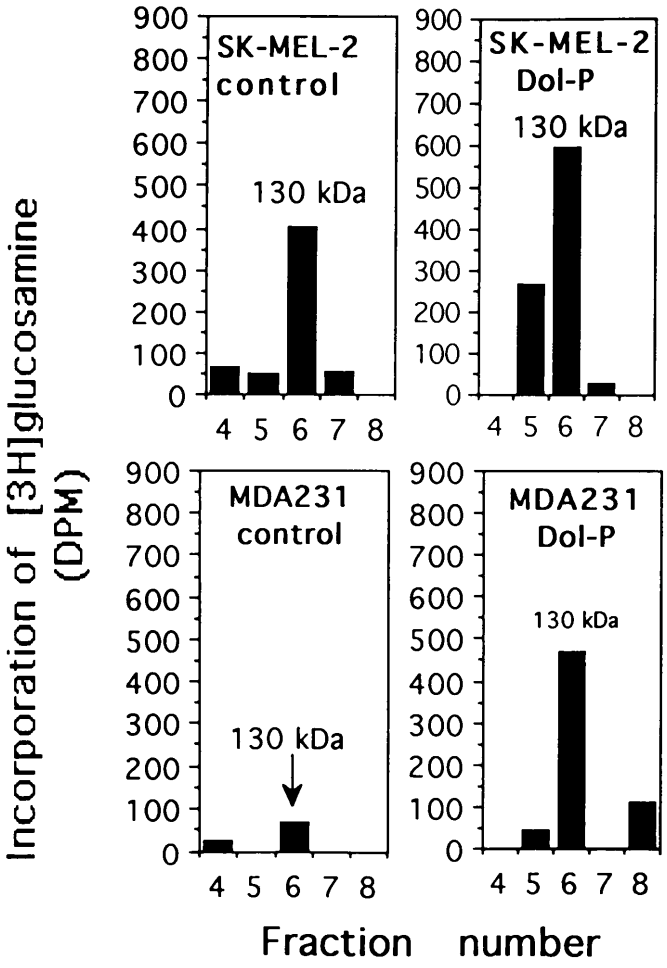

Fig. 5. N-linked glycosylation of IGF-1R in SK-MEL-2 and MDA231 cells. Effects of dolichyl phosphate. The cells were incubated in complete medium without (left panels) or with dolichyl phosphate (10 μg/ml) for 24 h. During the last 16 h of the incubation period the cells were labeled with [^{3}H]glucosamine (10 μCi/ml). Proteins from whole cells were then isolated and subjected to immunoprecipitation using αIR-3. The immunoprecipitates were run on SDS–PAGE and the radioactivity in gel slices was measured by scintillation counting. Fractions in the molecular weight region of the α-subunit of IGF-1R (130 kDa) are shown. Parallel analyses by Western blotting confirmed that the amounts of IGF-1R were essential equal in the different samples.

mine was purified by immunoprecipitation. As demonstrated in Figure 5 (left panels), the level of [^{3}H]glucosamine incorporation into IGF-1R was much lower in MDA231 compared to SK-MEL-2 cells. In contrast, there was no large difference between SK-MEL-2 and MDA231 in the *overall* N-linked glycosylation, as assayed by determining the incorporation of [^{3}H]glucosamine into acid-precipitable total proteins (in SK-MEL-2 12,800 ± 2600 DPM/mg protein and in MDA231 10,800 ± 1400 DPM/mg protein).

We now raised the question whether the level of dolichyl phosphate is of importance for the rate of N-linked glycosylation of IGF-1R. In a recent study we demonstrated that exogenous ^{3}H-labelled dolichyl phosphate was taken up by the cells and was incorporated into oligosaccharyl dolichyl phosphate complexes (Dricu *et al*., 1997b). This provides evidence that exogenous dolichyl phosphate can participate in N-linked glycosylation (Dricu *et al*.,1997b). As shown in Figure 5 (right panels), addition of dolichyl phosphate (a mixture of dol-P-16- dol-P-22) (10 μg/ml) to the breast cancer cells drastically increased N-linked glycosylation of IGF-1R, whereas it had only a moderate stimulatory effect in the melanoma cells.

We also demonstrate that the level of *de novo* synthesized dolichyl phosphate, assayed by measuring the incorporation of [^{3}H]mevalonate into dolichyl phosphate, is much lower in MDA231 than in the SK-MEL-2 cells (Figure 6). The biosynthesis of dolichyl-18 and dolichyl-19 phosphates was especially low in MDA231 cells (Figure 6). These data suggest that the comparatively low N-linked glycosylation of IGF-1R in breast

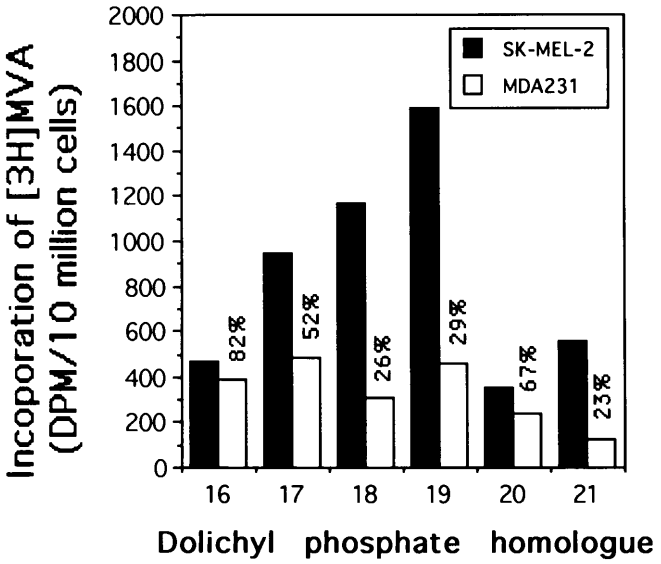

Fig. 6. Dolichyl phosphate synthesis in melanoma cells and breast cancer cells. SK-MEL-2 and MDA231 cells were labeled with [^{3}H]MVA for 24 h whereupon dolichyl phosphates were isolated and purified by reversed phase HPLC. The incorporation of [^{3}H]MVA into dolichyl phosphate homologues 16–21 is shown. The percentage values above the open bars indicate the relative homologue synthesis in MDA231 compared to that in SK-MEL-2 cells.

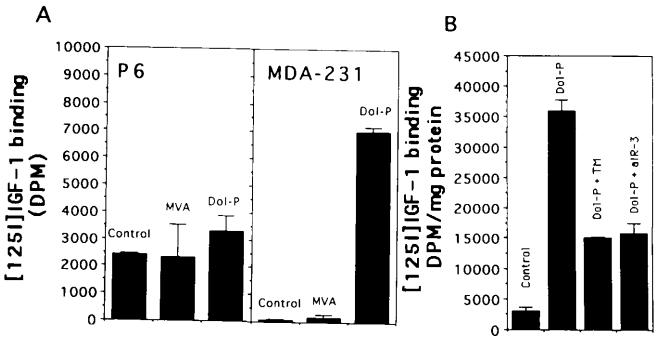

Fig. 7. IGF-1 binding in dolichyl phosphate–stimulated cells. (**A**) p6 cells (left panel) and MDA231 cells (right panel) were changed to new regular medium with or without MVA (0.77 mM) or dolichyl phosphate (a mixture of dol-P-16 - dol-P-22) (10 μg/ml). After a 24 h incubation period ^{125}I-IGF-1 binding was assayed. Mean values and SD values of duplicate determinations are shown. (**B**) MDA231 cells were treated with dolichyl phosphate (a mixture of dol-P-16 - dol-P-22) (10 μg/ml), or dolichyl phosphate + TM (10 μg/ml), or dolichyl phosphate + αIR-3 (1 μg/ml) for 24 h, after which they were assayed for ^{125}I-IGF-1 binding. Mean values and SD values of duplicate determinations are shown.

cancer cells might be due to a lack of *de novo*–synthesized dolichyl phosphate.

In Figure 7A (right panel) it is shown that addition of dolichyl phosphate (10 μg/ml) to MDA231 cells gives rise to a drastically increased IGF-1 binding activity. In contrast, addition of mevalonate had no effect. As compared to MDA231 cells, dolichyl phosphate did not significantly increase the binding activity in the p6 cells. There was no increase in IGF-1 binding in dolichyl phosphate–treated SK-MEL-2 either (data not shown). Whereas equivalent concentrations of mevalonate only caused a slight increase in IGF-1 binding, other isoprenoid lipids (i.e., cholesterol and coenzyme Q) had no measurable effect (data not shown). A similar experiment was also performed on Hs578T cells. Dolichyl phosphate, however, failed to increase the IGF-1 binding sites in this cell line (data not shown). This result is consistent with the previous observations that Hs578T cells express atypical IGF-1R (Peyrat and Bonneterre, 1992). In Figure 7B it is shown that the stimulatory effect of a 24 h treatment with dolichyl phosphate on IGF-1 binding was drastically counteracted by both TM (which decreases N-linked glycosylation and cell surface expression of IGF-1R) and αIR-3 (which blocks the IGF-1 binding site of the receptor).

In Figure 8A it is confirmed using Western blotting that dolichyl phosphate stimulates the translocation of the IGF-1R proteins to the plasma membrane in the breast cancer cells. Two independent experiments are shown. In Figure 8B the dose–response relationship between dolichyl phosphate and IGF-1 binding is demonstrated. A dolichyl phosphate concentration as low as 0.1 μg/ml was enough to elicit a drastic stimulatory effect (~50% of that obtained by a 100-fold higher dose). Higher doses than 10 μg/ml did not give any further stimulation of the IGF-1 binding (data not shown). In Figure 9A the stimulatory effect of dolichyl phosphate, compared to untreated cells, on the number of binding sites is illustrated by a Scatchard plot. As shown in Figure 9B, the dolichyl phosphate–stimulated increase in membrane-bound binding sites was associated with a substantial increase in tyrosine phosphorylation of the β-subunit (90 kDa) of IGF-1R. This means that the expressed receptors at the cell surface are biochemically active. Coincubation of the cells with αIR-3, however, resulted in a drastic decrease in receptor phosphorylation. The magnitude of this inhibition was of the same order as that on the IGF-1 binding (compare with Figure 7B).

We also analyzed the effects of three different dolichyl phosphate homologues (dol-P-17, -19, and -20), compared to the mixture, on IGF-1 binding in MDA231 cells. Hence, all three homologues stimulated IGF-1 binding drastically, comparable to the effect obtained by the dolichyl phosphate mixture (data not shown).

Finally, the effect of αIR-3 on the growth of dolichyl phosphate–stimulated breast cancer cells is shown. As distinguished from the case with control cells, αIR-3 blocked the growth of dolichyl phosphate–treated MDA231 (Figure 10). This result seems reasonable since the translocation of IGF-1R to the cell surface would make the cells dependent on the extracellular binding state.

Discussion

The level of IGF-1R expression has been shown to vary considerably among different breast cancer cell lines. Whereas estrogen receptor-positive BT-20 cells contained as many as 230 fmol binding sites/mg protein, estrogen receptor-negative MDA231 cells had only 7 fmol binding sites/mg protein (Peyrat and Bonneterre, 1992). In general there seems to be a correlation between the number of steroid receptors and the number of IGF-1 binding sites (Peyrat and Bonneterre, 1992). Since breast cancer cells have been shown to synthesize and secrete IGF-1 and IGF-2 (Minuto *et al.*, 1987; Huff *et al.*, 1988a,b), it is possible that the growth of these cells is controlled by autocrine IGF-1 pathways. However, whereas the blockade of the IGF-1 binding domain in the receptor using αIR-3 inhibited IGF-1-stimulated growth of breast cancer cells, this antibody did not suppress serum-free cell growth of MDA231 (Arteaga *et al.*, 1989; Arteaga, 1992). From

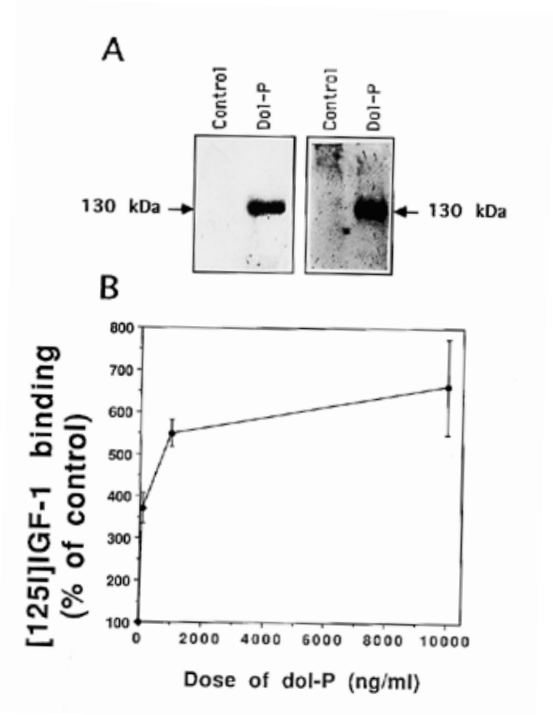

Fig. 8. Effect of dolichyl phosphate on expression of IGF-1R α-subunit at cell surface, and dose-response effects on IGF-1 binding. (**A**) MDA231 cells were shifted to new regular medium with or without dolichyl phosphate (a mixture of dol-P-16 - dol-P-22) (10 μg/ml). After 24 h the cells were harvested for isolation of plasma membrane proteins, whereupon Western blotting for analysis of the α-subunit of IGF-1R was performed. Detections from two separate experiments are shown. (**B**) MDA231 cells were treated with different concentrations of dolichyl phosphate (a mixture of dol-P-16 - dol-P-22) (0–10 μg/ml) for 24 h, after which ^{125}I-IGF-1 binding was assayed. Mean values ± SD of two experiments are illustrated.

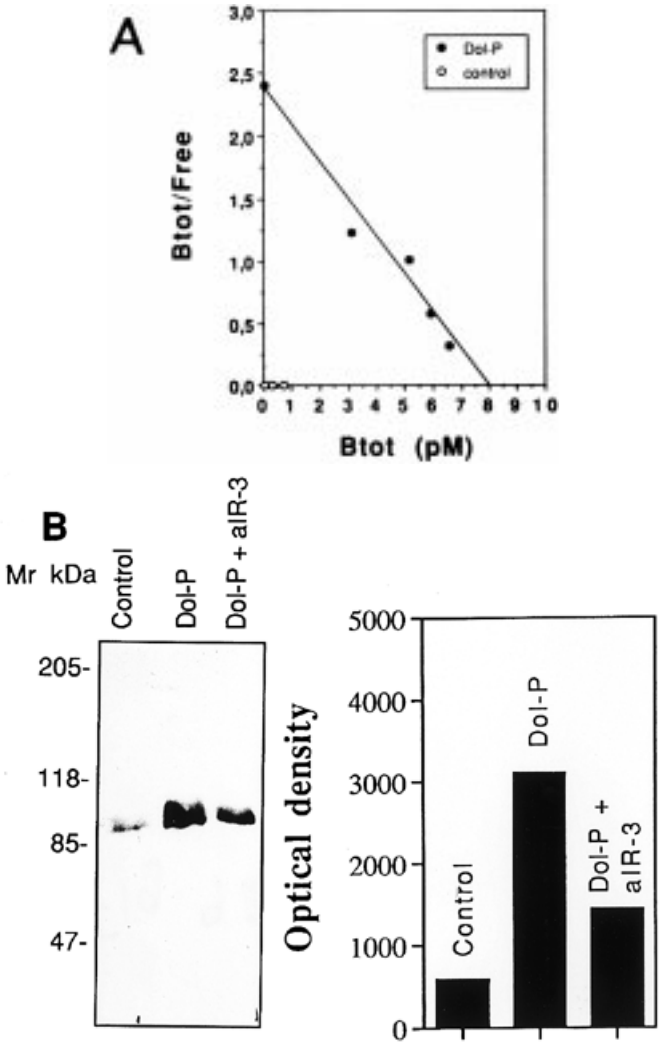

Fig. 9. (**A**) Scatchard plot analysis of dolichyl phosphate–stimulated cells. MDA231 cells remained either untreated (control) or were treated with dolichyl phosphate (a mixture of dol-P-16 - dol-P-22) (10 μg/ml) for 24 h. The cells were then incubated with ^{125}I-IGF-1 and different concentrations of unlabeled ligand (10–1000 ng/ml), after which Scatchard plot analysis was performed. The values are the means for duplicate determinations. (**B**) Autophosphorylation of IGF-1R β-subunit. MDA231 cells remained either untreated (control) or were treated with dolichyl phosphate (a mixture of dol-P-16 - dol-P-22) (10 μg/ml) without or with the presence of αIR-3 (1 μg/ml). After 24 h the cells were harvested for isolation of plasma membrane proteins, whereupon immunoprecipitation and Western blotting for analysis of tyrosine phosphorylated β-subunit of IGF-1R was performed (left section). Densitometric quantitation of the 90 kDa signals are shown in the right section. Arbitrary units are used. The experiment was repeated twice with similar results.

these data it has been suggested that growth of breast cancer cells is not dependent on autocrine IGF-1 loops. However, an alternative possibility is that *intracellular* IGF-1 loops exist. Such a pathway would make the serum-free growth of breast cancer cells resistant to αIR-3, since the intracellular receptors are not available for the antibody.

In the present study, we show that the low expression of IGF-1R at the cell surface in estrogen receptor-negative breast cancer cells is related to a low level of *de novo*–synthesized dolichyl phosphate and a low rate of N-linked glycosylation of IGF-1R. The melanoma cells, which have a high IGF-1R expression at the cell surface, exhibited a 6- to 7-fold higher synthesis of dolichyl phosphate from mevalonate and an 8-fold higher N-linked glycosylation of the IGF-1R α-subunit compared to MDA231 cells. That the availability of dolichyl phosphate is necessary for N-linked glycosylation of IGF-1R was demonstrated by adding exogenous dolichyl phosphate to the cells. Exogenous dolichyl phosphate increased both N-linked glycosylation and plasma membrane expression of IGF-1R drastically. The receptors stimulated by dolichyl phosphate to the plasma membrane were confirmed to express tyrosine kinase activity. It has been shown elsewhere that MDA231 cells express an insulin receptor and IGF-1R tyrosine kinase inhibiting activity (Constantino *et al*., 1993; Belfiori *et al*., 1996). However, based on our present results we can conclude that the receptors in the plasma membrane are biochemically active. The effect of dolichyl phosphate on IGF-1R translocation was found to be specific since addition of other isoprenes failed to stimulate the receptor expression. Taken together with our previous finding that exogenously added dolichyl phosphate can function as a carrier of oligosaccharides (Dricu *et al*., 1997b), our present data suggest that the stimulatory effect of dolichyl phosphate on expression of IGF-1R at the cell surface is mediated through an increased glycosylation of the α-subunit of IGF-1R. The reason why *de novo* synthesis of dolichyl phosphate is much lower in the breast cancer cells, compared to the melanoma cells, is not known. Substantial differences in dolichol biosynthesis between various malignant cell types have, however, been reported (Henry *et al*.,

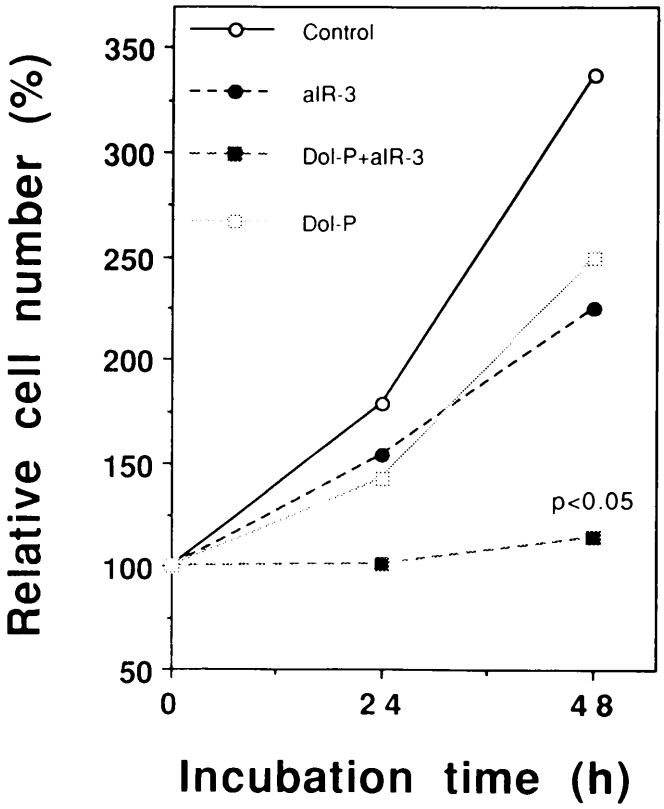

Fig. 10. Effect of dolichol phosphate–induced IGF-1R translocation on growth of αIR-3-treated breast cancer cells. MDA231 cells were shifted to new fresh control medium, or medium containing αIR-3 (1 μg/ml), or dolichyl phosphate (a mixture of dol-P-16 - dol-P-22) (10 μg/ml) + αIR-3 (1 μg/ml), or only dolichyl phosphate for 48 h. Cells were counted at every 24 h. The mean values of two determinations are shown. Statistical significance using Student's t-test is indicated.

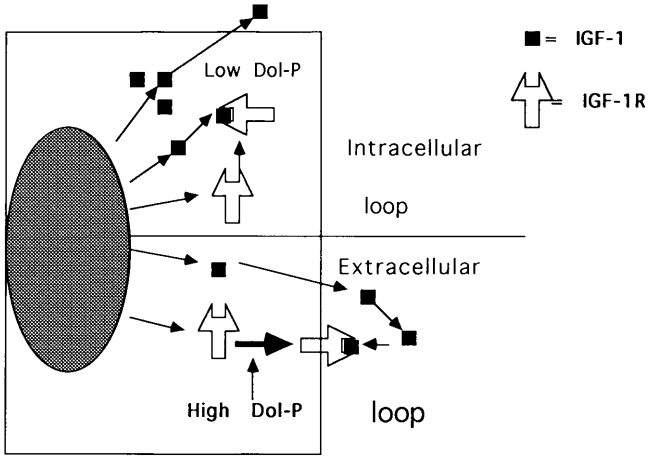

Fig. 11. A scheme showing two possible IGF-1 pathways in breast cancer cells, and the regulatory role of dolichyl phosphate.

1991). One explanation could involve differences in the function or specificity of enzymes involved in the dolichol biosynthetic pathway in different cell types. Mutant cell types synthesizing hypoglycosylated glycoproteins have been reported (Hart, 1992). In contrast to the differences in *de novo* synthesis of dolichyl phosphate and N-linked glycosylation of IGF-1R there was no significant difference in the rate of *overall* N-linked glycosylation between MDA231 and SK-MEL-2 cells. This suggests that the comparatively low rate of dolichyl phosphate biosynthesis in MDA231 is enough for maintaining an adequate glycosylation level of the bulk of glycoproteins, whereas some individual glycoproteins (like IGF-1R) require a higher supply of dolichyl phosphate.

By analyzing the binding of IGF-1 to solubilized IGF-1R, we could show that the retained intracellular receptors in MDA231 expressed binding activity. Because breast cancer cells produce IGF-1 and IGF-2 (which also binds to IGF-1R) the possibility of an intracellular autocrine IGF-1 pathway in estrogen-independent breast cancer cells is raised. Such a pathway would explain why the basal growth of MDA231 is not blocked by αIR-3 (Arteaga *et al.*, 1989; Arteaga, 1992). However, when IGF-1R was translocated to the plasma membrane following treatment with dolichyl phosphate, the growth of MDA231 cells was efficiently blocked by αIR-3. Taken together with our observation that translocated receptors are biochemically active, this suggests that treatment with dolichyl phosphate restores a IGF-1R growth pathway. In this context we could also demonstrate that treatment of the dolichyl phosphate–stimulated MDA231 cells with αIR-3 resulted in a decreased autophosphorylation of IGF-1R β-subunit. This result is important since it confirm that αIR-3 really acts as a IGF-1 antagonist in MDA231 cells. This has not to be the case in all cell types. In Chinese hamster ovary cells and NIH 3T3 cells transfected with cDNA for the human IGF-1R gene it was found that αIR-3 can act as an IGF-1 agonist (Steele-Perkins *et al.*, 1988; Kato *et al.*, 1993). Although αIR-3 seems to block IGF-1R function specifically in MDA-231 cells, we cannot exclude that this antibody may interfere with other mechanisms in the cells. As can be seen in Figure 1B,C, treatment with αIR-3 reduced growth rate, though only to a slight extent, in both MDA-231 and Hs578T. Since these cells express no (or few) and defective receptors at the cell surface, respectively, this effect most likely does not involve the IGF-1 pathway.

In conclusion our data suggest that the low plasma membrane expression of IGF-1R in estrogen-independent breast cancer cells is due to a low rate of *de novo* synthesis of dolichyl phosphate. Therefore, it is possible that mechanisms modulating the rate of dolichyl phosphate synthesis are critical for whether the cells use an intracellular or an extracellular IGF-1 pathway (Figure 11). Based on the results of the present study, we are not able to prove that the MDA231 cells contain functionally active intracellular IGF-1R. However, studies on this matter are in progress in our laboratory.

Materials and methods

Chemicals

A mouse monoclonal antibody (αIR-3) against the human IGF-1R was purchased from Oncogene Science, NY. A polyclonal IGF-1R antibody (N-20), a monoclonal EGF receptor antibody EGFR (528), and a mouse monoclonal antibody against phosphotyrosine (PY99) were from Santa Cruz Biotechnology Inc., Santa Cruz, CA. ^{125}I-IGF-1 (1630–2800 Ci/mmol) and R,S-[5–^{3}H]mevalonolactone (50 Ci/mmol) were obtained from New England Nuclear (via Dupont, Sweden). D-[6–^{3}H]Glucosamine (28.0 Ci/mmol) was from Amersham, UK. All other chemicals unless stated otherwise were from Sigma (St. Louis, MO).

Cells

The breast cancer cell lines MDA231, MCF-7, and Hs578T, and the human melanoma cell line SK-MEL-2 were obtained from American Type Culture Collection, Rockville, MD. The p6 cell line, Balb/c3T3 cells stably transfected with human IGF-1R cDNA (Pietrzkowski *et al.*, 1992), was kindly given to us by Dr. Renato Baserga (Thomas Jefferson University, Philadelphia, PA). Using immunostaining we could confirm that MDA231 lacks estrogen receptors (data not shown). Herewith MCF-7 cells (which highly express estrogen receptors) were used as a positive control.

Cell culture

MDA231 and Hs578T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum. SK-MEL-2 cells were cultured in Minimum Essential Medium supplemented with 10% fetal calf serum (FCS) and nonessential amino acids. The p6 cell line was cultured in DMEM containing 2% geneticin and 5% FCS.

The cells were grown in monolayers in tissue culture flasks maintained in a 95% air, 5% CO_2 atmosphere at 37°C in a humidified incubator. For experimental purposes cells were cultured in 35 mm, 60 mm, or 150 mm dishes. Cells were seeded at a density of 3000–5000 cells/cm^2, and the experiments were initiated when they had reached subconfluence.

Isolation of plasma membrane

Preparation of plasma membranes was performed essentially as described elsewhere (Gammeltoft, 1990). In brief, cells were harvested and homogenized in a buffer containing 0.32 M sucrose, 1 mM taurodeoxycholic acid, 2 mM $MgCl_2$, 1 mM EDTA, 25 mM benzamidine, 1 μg/ml bacitracin, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml soyabean trypsin inhibitor, and 10 μg/ml leupeptin. After a 10 min centrifugation at 600 × *g* (4°C) the pellet (containing unbroken cells, nuclei, and cytoskeleton) was discarded. The supernatant was then centrifuged at 17,300 × *g* for 30 min. The resulting pellet contained plasma membranes, and the supernatant represented the intracellular compartment containing the cytosol, ER, Golgi, and other organelles (Sheeler, 1981).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE)

Protein samples were dissolved in a sample buffer containing 0.0625 M Tris–HCl (pH 6.8), 20% glycerol, 2% SDS, bromophenol blue, and dithiothreitol. Samples corresponding to 150 μg cell protein were analyzed by SDS–PAGE with a 4% stacking gel and a 7.5% or 10% separation gel essentially according to the protocol of Laemmli (Laemmli, 1970). Molecular weight markers (Bio-Rad, Sweden) were run simultaneously. In one set of experiments the incorporation of [^{3}H]glucosamine into immunoprecipitated IGF-1R was measured by counting the radioactivity in gel slices. Hence 2 mm gel slices were put into scintillation vials and dissolved in 0.5 ml Soluene-350 (Canberra-Packard). After a 3 h incubation at 50°C, 8 ml of Hionic Fluor (Canberra-Packard) was added and the radioactivity was counted.

Determination of overall N-linked glycosylation

Total incorporation of D-[6–^{3}H]glucosamine (1 μCi/ml) into acid-stable glycoproteins was determined according to the description of Carson and Lennarz (1981). The radioactivity was normalized to protein content.

Immunoprecipitation of IGF-1R

The isolated cells were lysed in 10 ml ice-cold PBSTDS (made up using 100 ml 10× PBS with 10 ml of 100% Triton X-100, 5 g sodium deoxycholate, and 1 g sodium dodecyl sulfate in 1000 ml of deionized water) containing the aforementioned protease inhibitors; 15 ml Protein G Plus-Agarose and 1 μg αIR-3 was added to 1 ml of the cell lysate. After a 24 h incubation at 4°C on a rocker platform, the immunoprecipitates were collected by centrifugation in a microcentrifuge at 2500 r.p.m. for 15 min. The supernatant was discarded, whereupon the pellet was washed four times with 1 ml of PBSTDS. The material was then dissolved in sample buffer for SDS–PAGE.

Western blotting

Following SDS–PAGE the proteins were transferred overnight to nitrocellulose membranes (Hybond, Amersham) and then blocked for 1 h at room temperature in a solution of 5% (w/v) skimmed milk powder and 0.02% (w/v) Tween 20 in PBS, pH 7.5. Incubation with the IGF-1R antibody (N-20) or the antiphosphotyrosine antibody (PY99) was performed for 1 h at room temperature. This was followed by three washes with PBS and incubation with a biotinylated secondary antibody (Amersham) for 1 h. After a 15 min incubation with streptavidin-labeled horse peroxidase bands were detected (Hyperfilm-ECL, Amersham).

IGF-1R β-subunit autophosphorylation

Plasma membrane proteins were isolated and immunoprecipitated with αIR-3 as described above. The immunoprecipitate was then subjected to SDS–PAGE under reduced conditions and transferred to a Hybond membrane. The membrane was incubated with a phosphotyrosine antibody (PY99) (1:500), after which detection was performed.

^{125}I-IGF-1 binding assay

Cells growing in 35mm dishes were subjected to different treatments, after which they were rinsed twice with ice-cold PBS and once with binding buffer (1 mM Hepes pH 7.4, 1% BSA, 135 mM NaCl, 4.8 mM KCl, 1.7 mM $MgSO_4$, 2.5 mM $CaCl_2 \times 2H_2O$). Finally, each dish was incubated for 30 min at 20°C with 1 ml binding buffer containing 60,000 DPM of ^{125}I-labeled IGF-1. Thereafter, the cells were washed twice with PBS to remove unbound ligand and then lysed in a solution buffer (20 mM Hepes, 1% Triton-X, 10% glycerol, and 0.1% BSA), transferred to scintillation vials and counted in a scintillation counter. The nonspecific binding was determined by coincubation with unlabeled IGF-1 (1000 ng/ml).

Scatchard analysis was performed essentially as above except that the incubation with ^{125}I-IGF-1 was run at 4°C for 5 h. While the ^{125}I-IGF-1 concentration was constant the concentration of unlabeled ligand varied between 10 and 1000 ng/ml. The number of receptors/cell was calculated as described by Gammeltoft (1990).

Synthesis of dolichyl phosphate

Cells grown in 150 mm dishes were labeled with [^{3}H]mevalonate (10 μCi/ml) for 24 h. Isolation and purification of dolichyl

phosphate with reversed phase HPLC was performed essentially following the description of Adair and Keller (1985). The radioactivity in fractions corresponding to dolichyl phosphates (dol-P-16 - dol-P-22) was determined by scintillation counting and corrected for variation in the number of cells.

Analysis of IGF-1/IGF-1R gene expression

From the published cDNA sequences of the α-subunit of human IGF-1R (GenBank accession number X04434) and human IGF-1 (GenBank accession number X57025), oligonucleotide primers were designed using OLIGO Primer Analysis Software (National Biosciences, Inc., Plymouth, MN). Total RNA was isolated using RNeasy mini kit (Qiagen) according to the description of the manufacturer. Samples were first reverse-transcribed using the primers 5′-GCG GTA TTC AGC CTC CTC CTT C-3′ (position 2181) for IGF-1R mRNA and 5′-AAC GCC CAT CTT TTA AAT GTT ATC A-3′ (position 730) for IGF-1 mRNA. RT was performed using 1.5 μl of 20 mM/dNTP mix (Pharmacia Biotech), 20 U RNase inhibitor (Boehringer Mannheim), 16 μg albumin (BSA) (MBI Fermentas), 4.0 μl 5× first strand buffer (Gibco BRL Life Technologies), 2.0 μl 0,1 M DTT (Gibco), 200 U Superscript RT (Gibco), 3 μM primer, and 250 ng total RNA in a final volume of 20 μl. The reaction was performed at 42 °C for 60 min and at 95 °C for 5 min to inactivate the reverse transcriptase. The resulting cDNA was amplified by PCR using primers for IGF-1R: 5′-GCC CGA AGG TCT GTG AGG AAG AA-3′ (position 1028) and 5′-GGT ACC GGT GCC AGG TTA TGA TGA-3′ (position 1559) (Ullrich *et al*., 1986), and for IGF-1; 5′-GAG CCT GCG CAA TGG AAT AAA GTC-3′ (position 32) and 5′-CGG TGG CAT GTC ACT CTT CAC TC-3′ (position 644). The 50 μl PCR reaction solution consisted of 0.5 μl of 20 mM/dNTP mix (Pharmacia Biotech), 5 μl 10× PCR Buffer (Perkin Elmer), 2.5 mM $MgCl_2$ (Perkin Elmer), 5 μl cDNA from RT, 1 U AmpliTaq DNA Polymerase (Perkin Elmer), and 1.0 μM of each primer. Amplification was performed using a Perkin Elmer Thermal Cycler at 96 °C for 1 min, 60 °C for 30 s, and 72 °C for 30 s for 40 cycles and a final elongation for 10 min. The quality of RNA was confirmed using the §-actin primers 5′-CAT GCC ATC CTG CGT CTG GAC-3′ and 5′-CAC GGA GTA CTT GCG CTC AGG AGG-3′. Negative controls were included at every step of the RT-PCR preparations. A control without the RT step was included in order to check that the amplified products were generated from RNA. The PCR products were detected by ethidium bromide staining on a 2% TBE agarose gel.

Analysis of solubilized IGF-1R

Cells were scraped and suspended in a solubilization buffer (150 mM NaCl, 50 mM HEPES pH 7.4, 1% Triton X-100, 170 μg/ml phenylmethylsulfonyl fluoride, 1.0 μg/ml aprotinin, and 1.8 mg/ml bacitracin). The suspension was stirred for 1 h at 4 °C and then centrifuged at 100,000 × *g* for 1 h. The supernatant was applied to a wheat germ agglutinin–Sepharose column (0.5 × 5 cm) and washed once with 30 ml column buffer (30 mM NaCl, 30 mM HEPES pH 7.4, and 0.1% Triton X-100). The glycoproteins were eluted with 0.3 mM N-acetylglucosamine in the column buffer. The partially purified IGF-1R was then subjected to binding analysis as described by Gammeltoft (1990).

Cross-linking of IGF-1R

Cells growing in 6 cm dishes were rinsed three times with ice-cold PBS and then incubated with 0.35×10^6 DPM/dish of ^{125}I-IGF-1 in the aforementioned binding buffer with the addition of 1 μg/ml bacitracin for 2 h at 20 °C. Cross-linking of ^{125}I-IGF-1 to IGF-1R was then performed essentially as described by Gammeltoft *et al*. (1985).

Assay of cell growth and survival

Cell growth was measured by determining the number of cells attached to the plastic surface in duplicate 35 mm dishes. This was performed by microscopic counting of cells in several ink-marked areas on the dish bottom. By repeating the countings after specified time intervals, changes in the number of attached cells could be followed.

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Abbreviations

αIR-3, antibody against α-subunit of IGF-1R; dNTP, deoxynucleotide; Dol-P, dolichyl phosphate; ER, endoplasmic reticulum; IGF-1R, insulin-like growth factor-1 receptor; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PBSTDS, PBS containing Triton X-100, sodium deoxycholate, and sodium dodecyl sulfate; SDS, sodium dodecyl sulfate; TM, tunicamycin.

References

Adair,W.L. and Keller,K. (1985) Isolation of dolichol and dolichyl phosphate. *Methods Enzymol*., **111,** 201–215.

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

Arteaga,C.L. (1992) Interference of the IGF system as a strategy to inhibit breast cancer growth. *Breast Cancer Res. Treat*., **23,** 101–106.

Baserga,R. (1995) The insulin-like growth factor 1 receptor: a key to tumor growth? *Cancer Res*., **55,** 249–252.

Belfiori,A., Constantino,A., Frasca,F., Pandini,G., Mineo,R., Vigneri,P., Maddux,B., Goldfine,I.D. and Vigneri,R. (1996) Overexpression of membrane glycoprotein PC-1 in MDA-MB231 breast cancer cells is associated with inhibition of insulin receptor tyrosine kinase activity. *Mol. Endocrinol*., **10,** 1318–1326.

Carlberg,M. and Larsson,O. (1996) Stimulatory effect of PDGF on HMG-CoA reductase activity and N-linked glycosylation contributes to the increased expression of IGF-1 receptors in human fibroblasts. *Exp Cell Res*., **223,** 142–148.

Carlberg,M., Dricu,A., Blegen,H., Wang,M., Hjertman,M., Zickert,P., Höög,A. and Larsson,O. (1996) Mevalonic acid is limiting for N-linked glycosylation and translocation of the insulin-like growth factor-1 receptor to the cell surface. *J. Biol. Chem*., **271,** 17453–17462.

Carson,D.D. and Lennarz,W.J. (1981)Relationship of dolichol synthesis to glycoprotein synthesis during embryonic development. *J. Biol. Chem*., **256,** 4679–4686.

Constantino,A., Milazzo,G., Giorgino,F., Russo,P., Goldfine,I.D., Vigneri,R. and Belfiori,A. (1993) Insulin-resistent MDA-MB231 human breast cancer cells contain a tyrosine kinase inhibiting activity. *Mol. Endocrinol*., **7,** 1667–1676.

Dricu,A., Carlberg,M., Wang,M. and Larsson,O. (1997a) Inhibition of N-linked glycosylation using tunicamycin causes cell death in human malignant cells: role of down-regulation of the insulin-like growth factor-1 receptor in induction of apoptosis. *Cancer Res*., **57,** 543–548.

Dricu,A., Wang,M., Hjertman,M., Malec,M., Blegen,H., Wejde,J., Carlberg,M. and Larsson,O. (1997b) Mevalonate-regulated cell growth in human tumor cells: role of glycosylation of the IGF-1 receptor, prenylation of Ras and expression of c-myc. *Glycobiology*, **7,** 625–633.

Evan,G., Wyllie,A.H., Gilberts,C.S., Littlewood,T.D., Land,H., Brooks,M., Peen,L.Z. and Hancock,D.C. (1992) Induction of apoptosis in ribroblasts by c-myc protein. *Cell*, **69,** 119–128.

Gammeltoft,S. (1990) Peptide hormone action. A practical approach. In Siddle,K. and Hutton,J.C. (eds.), *Peptide Receptors.* Oxford University Press, Oxford, pp. 1–41.

Gammeltoft,S., Haselbacher,G.K., Humbel,R.E., Fehlmann,M. and Van Obberghen,E. (1985) Two types of receptors for insulin-like growth factors in mammalian brain. *EMBO J.*, **4,** 3407–3412.

Harrington,E.A., Bennet,M.R., Fanidi,A. and Evan,G.I. (1994) C-myc-induced apoptosis in fibroblasts is inhibited by specific cytokines. *EMBO J.*, **13,** 3286–3290.

Hart,G.W. (1992) Glycosylation. *Curr. Opin. Cell Biol.*, **4,** 1017–1023.

Henry,A., Stacpoole,P.W. and Allen,C.M. (1991) Dolichol biosynthesis in human malignant cells. *Biochem. J.*, **278,** 741–747.

Huff,K.K., Kaufman,D., Gabbay,K.H., Spencer,E.M., Lippman,M.E. and Dickson,R.B. (1988a) Secretion of an insulin-like growth factor-I-related protein by human breast cancer cells. *Cancer Res.*, **46,** 4613–4619.

Huff,K.K., Knabbe,C., Lindsey,R., Kaufman,D., Bronzert,D., Lippman,M.E. and Dickson,R.B.(1988b) Multihormonal regulation of insulin-like growth factor-I-related protein in MCF-7 human breast cancer cells. *Mol. Endocrinol.*, **2,** 200–208.

Kalebic,T., Tsokos,M. and Helman,L. (1994) *In vivo* treatment with antibody against the IGF-I receptor suppresses growth of human rhabdomyosarcoma and down regulates p34/cdc2. *Cancer Res.*, **54,** 5531–5534.

Kato,H., Faria,T.N., Stannard,B., Roberts,C.T.,Jr. and LeRoith,D. (1993) Role of tyrosine kinase activity in signal transduction by the insulin-like growth factor-1 (IGF-1) receptor. Characterization of kinase-deficient IGF-1 receptors and the action of an IGF-1-mimetic antibody (αIR-3). *J. Biol. Chem.*, **268,** 2655–2661.

Laemmli,U.K. (1970) Most commonly used discontinuous buffer systems for SDS electrophoresis. *Nature*, **227,** 680–685.

Massagué,J. and Czech,M.P. (1992) The subunit structures of two distinct receptors for insulin-like growth factors I and II and their relationship to the insulin receptor. *J. Biol. Chem.*, **257,** 5038–5045.

Minuto,F., Del Monte,P., Barreca,A., Nicolin,A. and Giordano,G. (1987) Partial characterization of somatomedin C-like immunoreactivity secreted by breast cancer cells *in vitro*. *Mol. Cell. Endocrinol.*, **54,** 179–184.

Peyrat,J.P. and Bonneterre,J. (1992) Type 1 IGF-1 receptor in human breast diseases. *Breast Cancer Res. Treat.*, **22,** 59–67.

Pietrzkowski,Z., Lammers,R., Carpenter,G., Soderquist,A.M., Limardo,M., Philiphs,P.D. and Baserga,R. (1992) Constitutive expression of IGF-1 and IGF-1 receptor abrogates all requirements for exogenous growth factors. *Cell Growth Differ.*, **3,** 199–205.

Prager,D., Li,H.L., Asa,S. and Melmed,S. (1994) Dominant negative inhibition of tumorigenesis *in vivo* by human insulin-like growth factor mutant. *Proc. Natl. Acad. Sci. USA*, **91,** 2181–2185.

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

Resnicoff,M., Abraham,D., Yutanawiboonchai,W., Rotman,H., Kajstura,J., Rubin,R., Zoltick,P. and Baserga,R. (1995) The insulin-like growth factor I receptor protects tumor cells from apoptosis *in vivo*. *Cancer Res.*, **55,** 2463–2469.

Sell,C., Baserga,R. and Rubin,R. (1995) Insulin-like growth factor I (IGF-I) and the IGF-I receptor prevent etoposide-induced apoptosis. *Cancer Res.*, **55,** 303–307.

Shapiro,D.N., Jones,B.G., Shapiro,L.H., Dias,P. and Houghton,P.J. (1994) Antisense-mediated reduction in insulin-like growth factor 1 receptor expression supresses the malignant phenotype of human alveolar rhabdomyosarcoma. *J. Clin. Invest.*, **94,** 1235–1242.

Sheeler,P. (1981) *Centrifugation in Biology and Medical Science*. Wiley, New York.

Steele-Perkins,G., Turner,J., Edman,J.C., Hari,J., Pierce,S.B., Stover,C., Rutter,W.J. and Roth,R.A. (1988) Expression and characterization of a functional human insulin-like growth factor 1 receptor. *J. Biol. Chem.*, **263,** 11486–11492.

Ullrich,A., Gray,A., Tam,A.W., Yang-Feneg,T., Tsubokawa,M., Collins,C., Henzel,W., Le Bon,T.,Kathuria,S., Chen,E., Jacobs,S., Francke,U., Ramachandran,J. and Fujita-Yamaguchi,Y. (1986) Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. *EMBO J.*, **5,** 2503–2512.