

Retinoic acid mediates degradation of IRS-1 by the ubiquitin–proteasome pathway, via a PKC-dependant mechanism

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Insulin receptor substrate-1 (IRS-1) mediates signaling from the insulin-like growth factor type-I receptor. We found that all-*trans* retinoic acid (RA) decreases IRS-1 protein levels in MCF-7, T47-D, and ZR75.1 breast cancer cells, which are growth arrested by RA, but not in the RA-resistant MDA-MB-231 and MDA-MB-468 cells. Based on prior reports of ubiquitin-mediated degradation of IRS-1, we investigated the ubiquitination of IRS-1 in RA-treated breast cancer cells. Two proteasome inhibitors, MG-132 and lactacystin, blocked the RA-mediated degradation of IRS-1, and RA increased ubiquitination of IRS-1 in the RA-sensitive breast cancer cells. In addition, we found that RA increases serine phosphorylation of IRS-1. To elucidate the signaling pathway responsible for this phosphorylation event, pharmacologic inhibitors were used. Two PKC inhibitors, but not a MAPK inhibitor, blocked the RA-induced degradation and serine phosphorylation of IRS-1. We demonstrate that RA activates PKC- δ in the sensitive, but not in the resistant cells, with a time course that is consistent with the RA-induced decrease of IRS-1. We also show that: (1) RA-activated PKC- δ phosphorylates IRS-1 *in vitro*, (2) PKC- δ and IRS-1 interact in RA-treated cells, and (3) mutation of three PKC- δ serine sites in IRS-1 to alanines results in no RA-induced *in vitro* phosphorylation of IRS-1. Together, these results indicate that RA regulates IRS-1 levels by the ubiquitin–proteasome pathway, involving a PKC-sensitive mechanism.

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Introduction

The family of insulin receptor substrate (IRS) proteins (IRS 1–4) function as the central substrates in the insulin

and insulin-like growth factor type-I (IGF-I) receptor signal transduction pathways (Sun *et al.*, 1991; Lavan *et al.*, 1997). Specifically, IRS-1 functions as a critical scaffolding protein between the activated IGF-IR and various downstream signaling pathways, including the PI3-kinase/AKT and MAPK pathways (Shepherd *et al.*, 1998; Burks and White, 2001). Among its many functions, this protein has been shown to regulate cell proliferation and survival (White, 1997). In hematopoietic cells, IRS-1 is an essential regulator of proliferation in response to insulin (Wang *et al.*, 1993), and in NIH 3T3 cells, cellular transformation can be directly induced by overexpressing IRS-1 (Ito *et al.*, 1996). Several studies have highlighted the importance of IRS-1 in breast cancer pathogenesis: IRS-1 overexpression in breast cancer cells causes a loss of estrogen-dependant growth (Surmacz and Burgaud, 1995), high levels of IRS-1 in human breast tumors correlate with increased disease recurrence (Rocha *et al.*, 1997; Lee *et al.*, 1999), constitutive IRS-1 signaling exists in breast tumors (Chang *et al.*, 2002), and expression of dominant-negative or antisense IRS-1 vectors in breast cancer cells decreases their transformation potential (Ando *et al.*, 1998; Chang *et al.*, 2002). This suggests that we may develop molecular strategies targeting IRS-1 by understanding the mechanisms controlling its expression and turnover.

Previous studies have suggested that the expression of IRS-1 can be regulated at the level of transcription and proteolysis. 17- β -Estradiol (E2) increases IRS-1 levels by increasing mRNA and protein levels (Lee *et al.*, 1999; Molloy *et al.*, 2000; Gonzalez *et al.*, 2001), while antiestrogens decrease IRS-1 mRNA and/or protein levels (Salerno *et al.*, 1999; Chan *et al.*, 2001). Glucocorticoids also reportedly decrease the levels of IRS-1 mRNA and protein (Turnbow *et al.*, 1994; Buren *et al.*, 2002). Using various cellular systems, including MCF-7 breast cancer cells, it has been shown that prolonged treatment with IGF-I and high concentrations of insulin can induce degradation of IRS-1 and IRS-2 via the ubiquitin–proteasome pathway (Sun *et al.*, 1999; Lee *et al.*, 2000; Zhang *et al.*, 2000; Haruta *et al.*, 2000).

Retinoids, including all-*trans*-retinoic acid (RA), have been shown to induce G1 arrest and differentiation in

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several cancer cell types by regulating various cellular factors. We previously showed that RA-mediated growth arrest of MCF-7 cells involves a decrease in IRS-1 protein levels (del Rincón *et al.*, 2003), and others have associated G1 arrest of bronchial epithelial cells with an RA-mediated decrease of cyclin D1 protein levels (Langenfeld *et al.*, 1997; Boyle *et al.*, 1999) and RA-mediated differentiation of F9 embryonal carcinoma cells with a decrease in the protein levels of the transcriptional coactivator, p300 (Iwao *et al.*, 1999; Brouillard and Cremisi, 2003). Although the past two decades have focused on examining transcriptional mechanisms linked to the pleiotropic effects of RA, the importance of post-translational modification of various proteins has been recently highlighted. RA has been shown to induce ubiquitination of a number of proteins, including cyclin D1 (Spinella *et al.*, 1999), p300 (Brouillard and Cremisi, 2003), and Skp2 (Dow *et al.*, 2001), and IRS-1 is known to be conjugated by ubiquitin (Lee *et al.*, 2000). However, the ubiquitination of IRS-1 by RA has not been reported. Therefore, we tested the

hypothesis that RA-mediated growth inhibition of breast cancer cells is associated with proteolytic degradation of IRS-1 by the ubiquitin–proteasome pathway.

Results

Regulation of IRS-1 protein levels in MCF-7 cells and other retinoid-responsive breast cancer cell lines

Consistent with our previous findings, exposure of MCF-7 cells to 1 μ M RA for 72 h in serum-free media (SFM) results in a dose-dependant decrease in IRS-1 protein levels (Figure 1a) (del Rincón *et al.*, 2003). Previous studies have shown that antiestrogens and glucocorticoids alter IRS-1 levels (Turnbow *et al.*, 1994; Salerno *et al.*, 1999; Chan *et al.*, 2001; Buren *et al.*, 2002); thus, we investigated whether other nuclear receptor-selective ligands would induce a similar decrease in IRS-1 levels. As expected, treatment of MCF-7

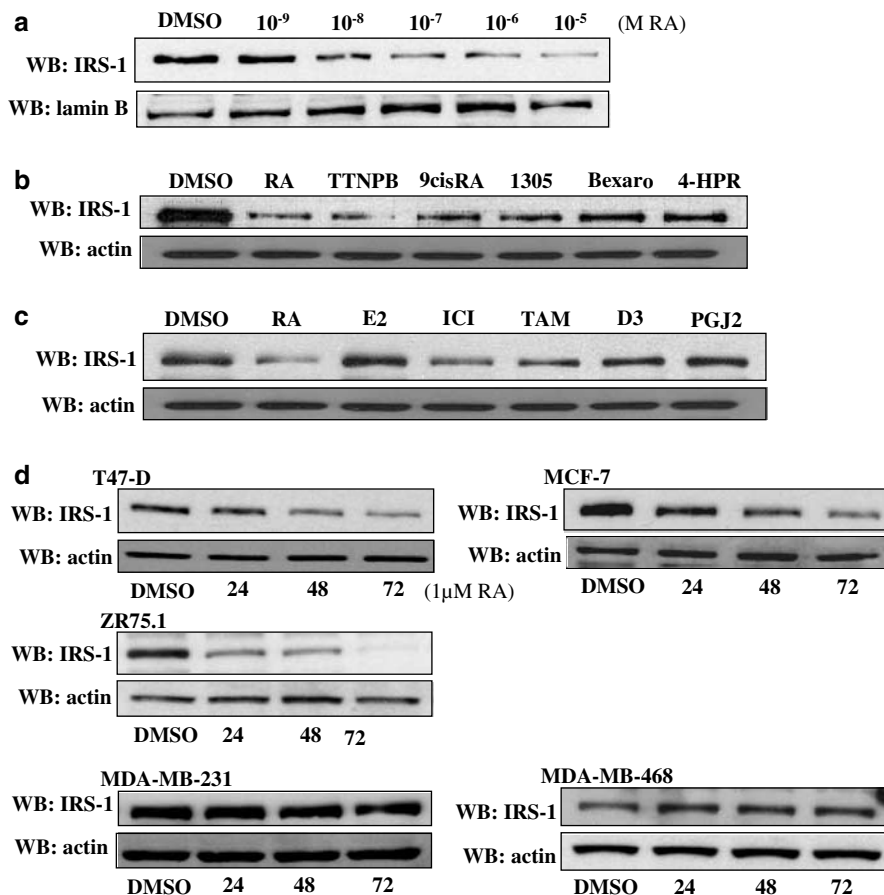


Figure 1 RA decreases IRS-1 protein levels in retinoid-sensitive breast cancer cell lines. Western blotting (WB) was used to determine the expression level of IRS-1 in (a)–(d). (a) MCF-7 cells were treated for 72 h in serum-free media (SFM) with increasing doses of RA (10⁻⁹, 10⁻⁸, 10⁻⁷, 10⁻⁶, 10⁻⁵ M). Lamin B was used as a loading control. (b) MCF-7 cells were treated with various retinoids: TTNPB, 9cisRA, LGD1305 (1305), Bexarotene (Bexaro), *N*-(4-hydroxyphenyl)retinamide (4-HPR) for 72 h in SFM. (c) MCF-7 cells were treated with various ligands: 17- β -estradiol (E2), tamoxifen (Tam), ICI 182780 (ICI), 1,25-dihydroxyvitamin D3 (D3), and 15-deoxy-delta 12,14-prostaglandin J2 (PGJ2) for 72 h in SFM. β -Actin (actin) was used as a loading control. (d) Various breast cancer cell lines (T47-D, MCF-7, ZR75.1, MDA-MB-231, MDA-MB-468) were treated with 1 μ M RA for 24, 48, and 72 h in SFM

cells with Tamoxifen and ICI 182780 results in decreased IRS-1 levels (Figure 1c). We also observed a decrease with RAR- α (TTNPB), RXR-selective ligands (LGD1305 and Bexarotene), and *N*-(4-hydroxyphenyl)-retinamide (4-HPR) (Figure 1b), while no significant effect was observed after treatment with the vitamin D3 selective ligand (1,25-dihydroxyvitamin D3) or the PPAR-selective ligand (15-deoxy-delta 12,14-prostaglandin J2) for 3 days (Figure 1c).

Although the mechanism of RA-mediated growth inhibition is unclear, it has generally been found that RA inhibits the growth of breast cancer cells that are ER-positive (MCF-7, T47-D, ZR75.1), while having minimal effects on breast cancer cells that are ER-negative (MDA-MB-231, MDA-MB-468) (van der Burg *et al.*, 1993; Rosenauer *et al.*, 1998). We hypothesized that regulation of IRS-1 levels by RA would correlate with RA-mediated growth inhibition of breast cancer cells. We treated RA-sensitive and RA-resistant breast cancer cell lines with 1 μ M RA for 24, 48 and 72 h in SFM. In support of our hypothesis, the RA-sensitive cells responded to 1 μ M RA treatment with a similar decrease in IRS-1 protein levels as MCF-7 cells, while the two RA-resistant cell lines did not (Figure 1d).

RA induces a post-translational modification of IRS-1 in MCF-7 cells

Previous studies have suggested that IRS-1 can be regulated at the transcriptional level (Lee *et al.*, 1999; Molloy *et al.*, 2000; Huang *et al.*, 2002). MCF-7 cells were treated with 10 μ M RA for 24, 48 and 72 h, total RNA was extracted and IRS-1 mRNA expression was examined by Northern blot, wherein the 8.5 kb transcript is shown (Figure 2a, top panel). Using RT-PCR, we observed no change in IRS-1 mRNA expression at any of the time points examined in the presence of 1 or 10 μ M RA, however, ICI 182780 was shown to decrease IRS-1 mRNA levels, consistent with previously published reports (Salerno *et al.*, 1999) (Figure 2a, bottom panel).

Recently, IRS-1 has been shown to translocate to the nuclei in various cellular backgrounds stimulated with insulin or IGF-I (Boylan and Gruppuso, 2002; Tu *et al.*, 2002; Sun *et al.*, 2003). Since the regulation of IRS-1 nuclear/cytoplasmic trafficking is not a well-understood process, we examined whether the RA-mediated decrease in IRS-1 protein levels might involve the regulation of IRS-1 localization. We prepared cytosolic and nuclear extracts from MCF-7 cells grown in serum-free medium with or without 1 μ M RA for 72 h. Under these conditions, there did not appear to be any redistribution of IRS-1 into the nucleus (Figure 2b).

Based on these results, we hypothesized post-transcriptional effect of RA on the synthesis or degradation rate of IRS-1. We first examined whether the decline in IRS-1 protein in MCF-7 cells treated with RA results from a decrease in the rate of IRS-1 synthesis. Using pulse-labeling with [³⁵S]methionine, we measured the synthesis rate of IRS-1 in MCF-7 cells pretreated with RA. The rate of IRS-1 synthesis was similar in cells

treated with DMSO vehicle or RA, suggesting that this is not the mechanism by which RA alters IRS-1 protein levels (Figure 2c). We then measured the rate of degradation of IRS-1 in the presence of DMSO or RA by pulse-chase analysis and found that RA-treated MCF-7 cells showed an accelerated rate of IRS-1 degradation compared to those cells treated with DMSO alone (Figure 2d). This suggests that RA activates a proteolytic pathway in MCF-7 cells that may be responsible for the degradation of IRS-1.

Effects of proteasome and protease inhibitors on degradation of IRS-1 by RA in breast cancer cells

Previous studies have reported that the levels of IRS-1 can be regulated by IGF-I and insulin at the protein level via the ubiquitin-proteasome pathway (Sun *et al.*, 1999; Lee *et al.*, 2000; Zhang *et al.*, 2000; Zhande *et al.*, 2002). We therefore tested whether the effects of RA on IRS-1 were mediated via a similar mechanism. The proteasome inhibitors lactacystin and MG-132 are reagents that inhibit the activity of the 26S proteasome, causing the accumulation of ubiquitinated proteins otherwise degraded by the ubiquitin-proteasome pathway. As shown in Figure 3a the decrease in IRS-1 levels in MCF-7 cells treated with 10 μ M RA for 48 h was rescued by the addition of lactacystin to the culture medium for the last 12 h of RA treatment. Similarly, MG-132 reversed the RA-mediated decline in IRS-1 levels (Figure 3b). Cyclin D1 is a protein known to be regulated by RA-mediated ubiquitination and subsequent proteolysis (Langenfeld *et al.*, 1997; Spinella *et al.*, 1999; Dow *et al.*, 2001). In Figure 3b, we show a similar rescue of cyclin D1 levels by MG-132 in RA-treated MCF-7 cells.

We next assessed whether IRS-1 levels could be restored by the addition of proteasome inhibitors in other RA-sensitive breast cancer cells. In the retinoid-responsive breast cancer cell lines T47-D and ZR75.1, we added MG-132 for the last 12 h of a 48-h 10 μ M RA treatment and observed a restoration of IRS-1 levels (Figure 3c). We further demonstrate the effect of RA on IRS-1 by immunofluorescence in T47-D cells (Figure 3d). Consistent with our Western blot data, treatment with RA for 48 h decreased IRS-1 protein staining, and treatment with MG-132 for the last 12 h of RA treatment blocked this effect. It has been reported that IRS-2 is regulated at the post-translational level via increased protein ubiquitination (Hirashima *et al.*, 2003; Morelli *et al.*, 2003); however, we failed to observe any regulation in the protein expression of IRS-2 in any of the breast cancer cell lines treated with RA (data not shown). Although MG-132 is an inhibitor of the proteasome, it also inhibits calpains, calcium-activated cysteine proteases. Furthermore, some studies have shown that IRS-1 degradation can be mediated via calpains (Smith *et al.*, 1993), so we assessed whether RA induces a decline in IRS-1 levels via a mechanism involving calpains.

In our system, the addition of the cell-permeable calpain inhibitor, calpeptin, could not restore IRS-1

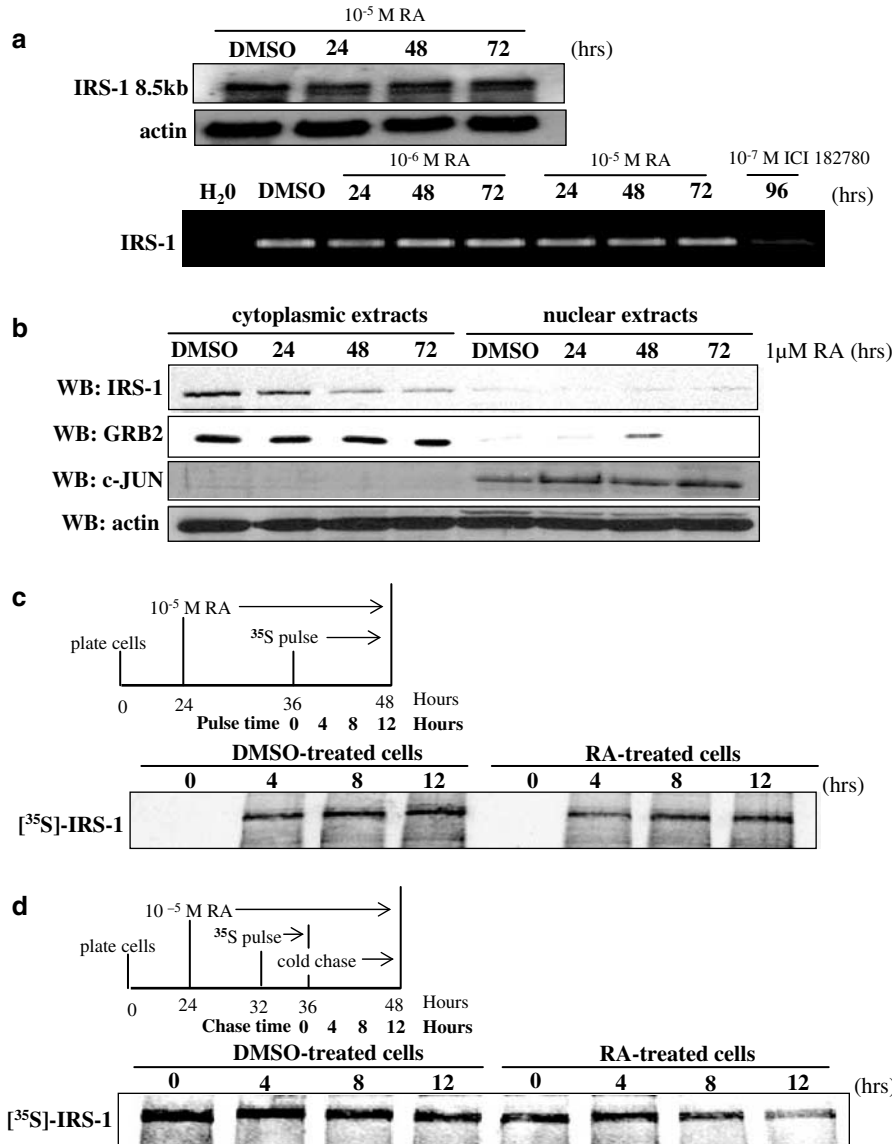


Figure 2 RA induces a post-translational modification of IRS-1 in MCF-7 cells. **(a)** MCF-7 cells were treated with 10⁻⁵ M RA in SFM for 24, 48, or 72 h and the expression of IRS-1 mRNA was evaluated by Northern blot (top panel). Actin was used to ensure equal loading. RT-PCR was used to detect IRS-1 levels in MCF-7 cells treated with 10⁻⁶ M or 10⁻⁵ M RA, or 10⁻⁷ M ICI 182780 (bottom panel). All data are representative of four independent experiments. **(b)** Subcellular fractionation was used to examine the expression of IRS-1 in cytoplasmic and nuclear protein lysates obtained from MCF-7 cells treated with RA for 24, 48, and 72 h. To control for the purity of the fractions, the levels of a nuclear protein (c-Jun), and a cytoplasmic protein (Grb2) were assessed by stripping and reprobings membranes. **(c)** The synthesis rate of IRS-1 in MCF-7 cells treated for 24 h in the presence of DMSO or 10⁻⁵ M RA was determined by pulse labeling with [³⁵S]methionine. IRS-1 abundance was analysed at 0, 4, 8, and 12 h time intervals after the pulse. **(d)** The degradation rate of IRS-1 in MCF-7 cells treated for 24 h in the presence of DMSO or 10⁻⁵ M RA was determined by pulse-chase analysis with [³⁵S]methionine. IRS-1 abundance was analysed at 0, 4, 8, and 12 h time intervals after the chase

levels (data not shown). This suggests that RA does not decrease IRS-1 levels via activation of a calpain-dependent pathway, but rather it may be increasing proteasomal activity.

RA enhances ubiquitination of IRS-1 in breast cancer cells

Proteasomal degradation of proteins involves the prior conjugation of ubiquitin to the targeted protein. We

examined whether ubiquitin-IRS-1 complexes could be formed in breast cancer cells treated with RA. IRS-1 immunoprecipitation and ubiquitin Western blotting revealed that the level of ubiquitin-IRS-1 conjugates is augmented in MCF-7 cells treated with RA and MG-132 compared to either agent alone (Figure 4a). This result was supported by cotransfection experiments using expression vectors for flag-IRS-1 and HA-Ub in MCF-7 cells. The cells were transfected with both

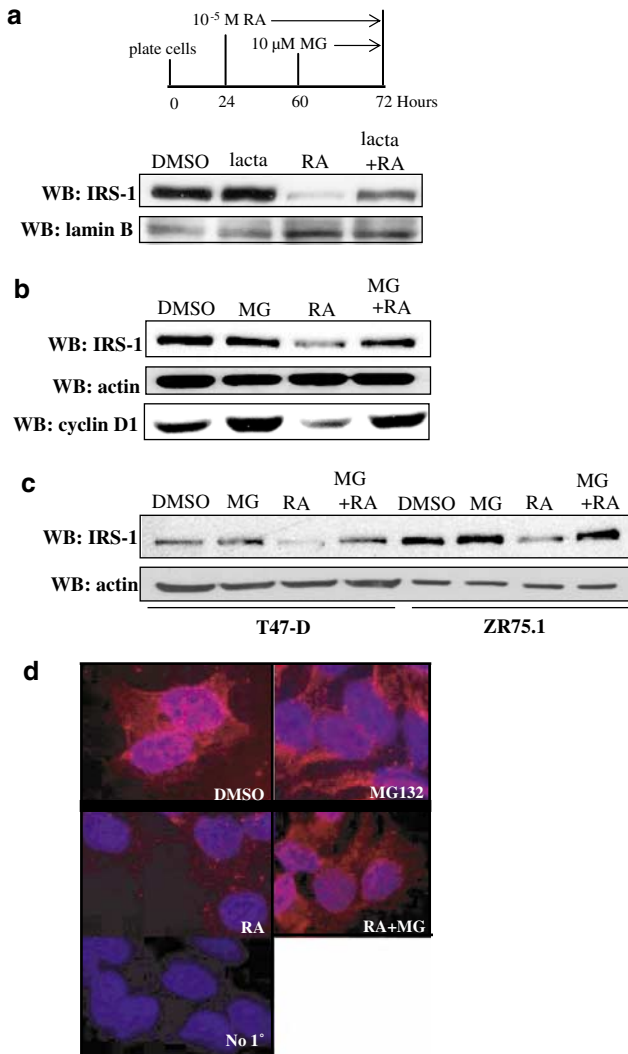


Figure 3 Proteasome inhibitors block the RA-mediated decrease in IRS-1 protein levels in retinoid-sensitive breast cancer cells. WB was used to determine the expression level of IRS-1 in (a)–(c). (a) MCF-7 cells were treated with DMSO or 10^{-5} M RA for 48 h in SFM. 10^{-5} M Lactacystin (lacta) was added for the last 12 h of RA treatment. (b) MCF-7 cells were treated with DMSO or 10^{-5} M RA for 48 h in SFM. 10^{-5} M MG-132 (MG) was added for the last 12 h of RA. Cyclin D1 levels were detected by WB. The data depicted are representative of at least three independent experiments. (c) T47-D and ZR75.1 cells were treated with DMSO or 10^{-5} M RA for 48 h in SFM. 10^{-5} M MG was added for the last 12 h of RA treatment. (d) T47-D cells were treated with DMSO or 10^{-5} M RA for 48 h in SFM. 10^{-5} M MG was added for the last 12 h of RA treatment. Immunofluorescence was used to detect IRS-1 proteins (red fluorescence) in cytoplasm of T47-D cells. DAPI staining revealed the nucleus (blue fluorescence). Similar results were repeated in MCF-7 cells (data not shown)

vectors and treated with either $10 \mu\text{M}$ RA or 10 nM IGF-I for 24 h, with or without the addition of MG-132 for the last 12 h of RA treatment. To show that IRS-1-ubiquitin conjugates bound more effectively in the presence of RA, these cells were lysed and the lysate immunoprecipitated

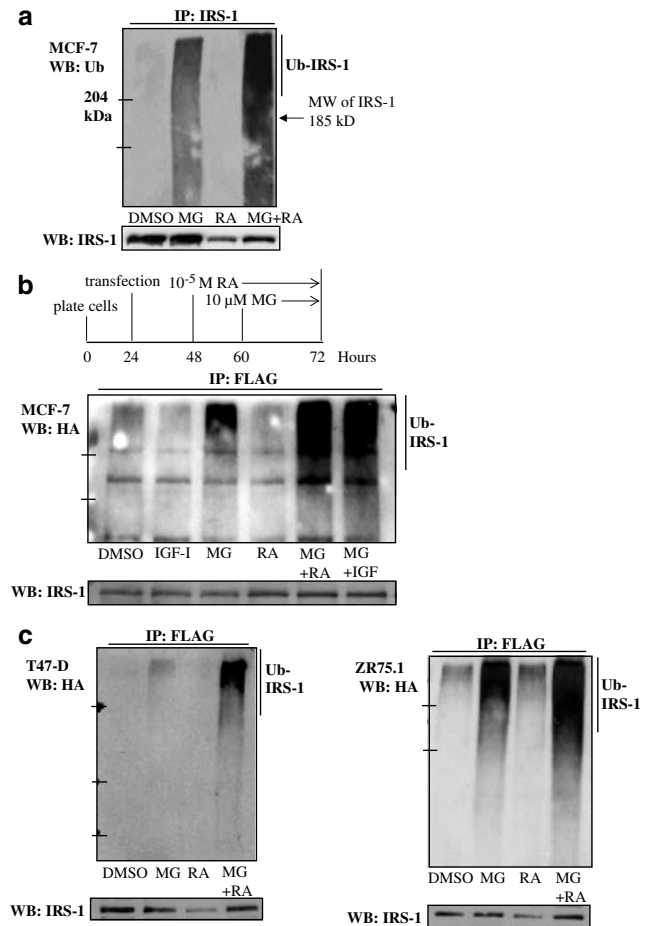


Figure 4 RA enhances ubiquitination of IRS-1 in breast cancer cells. (a) MCF-7 cells were treated with DMSO or 10^{-5} M RA for 48 h in SFM. 10^{-5} M MG-132 was added for the last 12 h of RA treatment. IRS-1 was immunoprecipitated (IP) from protein lysates, and the level of ubiquitination was evaluated by WB with an anti-ubiquitin (Ub) antibody. The same membrane was stripped and reprobed for IRS-1. (b) MCF-7 cells were transiently transfected with HA-Ub and flag-IRS-1 constructs. Cells were then treated in SFM for 24 h with 10 nM IGF-I or 10^{-5} M RA. 10^{-5} M MG-132 was added for the last 12 h of RA or IGF-I treatment. FLAG was immunoprecipitated (IP) from protein lysates, and the level of flag-IRS-1/HA-Ub interaction was evaluated by WB with an anti-HA (HA) antibody. The same membrane was stripped and reprobed for IRS-1. (c) T47-D and ZR75.1 cells were transfected and treated as described in (b)

with anti-flag antibodies and immunoblotted with anti-HA antibodies (Figure 4b). RA and MG-132 induced a marked increase in the association of flag-IRS-1 and Ha-Ub compared to MG-132 alone (Figure 4b, compare lanes 3 and 5). Furthermore, consistent with prior reports, IGF-I and MG-132 also augmented the level of detectable IRS-1-Ub conjugates in MCF-7 cells compared to MG-132 alone (Figure 4b, compare lanes 3 and 6) (Lee *et al.*, 2000). In two other RA-sensitive breast cancer cell lines, we found a similar dramatic increase in IRS-1-ubiquitin conjugates upon treatment with RA and MG-132 (Figure 4c). Taken together, these results show that RA enhances the ubiquitination of IRS-1.

Inhibiting the PKC pathway blocks the RA-mediated phosphorylation and degradation of IRS-1

Recent studies have shown that serine/threonine phosphorylation of IRS-1 signals its degradation, suggesting a requirement for the activation of specific kinases to regulate the levels of IRS-1. In support of this hypothesis, we find that RA-treated MCF-7 cells grown in 10% FBS have a greater and more rapid decrease in IRS-1 protein levels than when grown in serum-free media (Figure 5a). Consistent with the activation of a serine/threonine kinase in breast cancer cells treated with RA, we observe an increase in the total serine phosphorylation of IRS-1 (Figure 5b). Based on these data, we investigated the role of specific signaling pathways in mediating the

RA-induced decline in IRS-1 protein levels. Using a chemical inhibitor approach, the breast cancer cell lines, MCF-7, T47-D, and ZR75.1, were pretreated for 60 min with inhibitors of the MAPK (PD98059) and PKC (Rottlerin and GF109203X) signaling cascades and then treated for an additional 48 h with RA in the presence of the inhibitors. The decrease in IRS-1 protein levels could be inhibited in all of the breast cancer cells by treatment with Rottlerin and GF109203X, but not with PD98059 (Figure 5c). Consistent with this result, we also observed a block in the RA-mediated increase in serine phosphorylation of IRS-1 when the breast cancer cells were cotreated with Rottlerin (Figure 5d). These results suggest that a PKC-sensitive pathway is involved in the RA-mediated decline in IRS-1 levels.

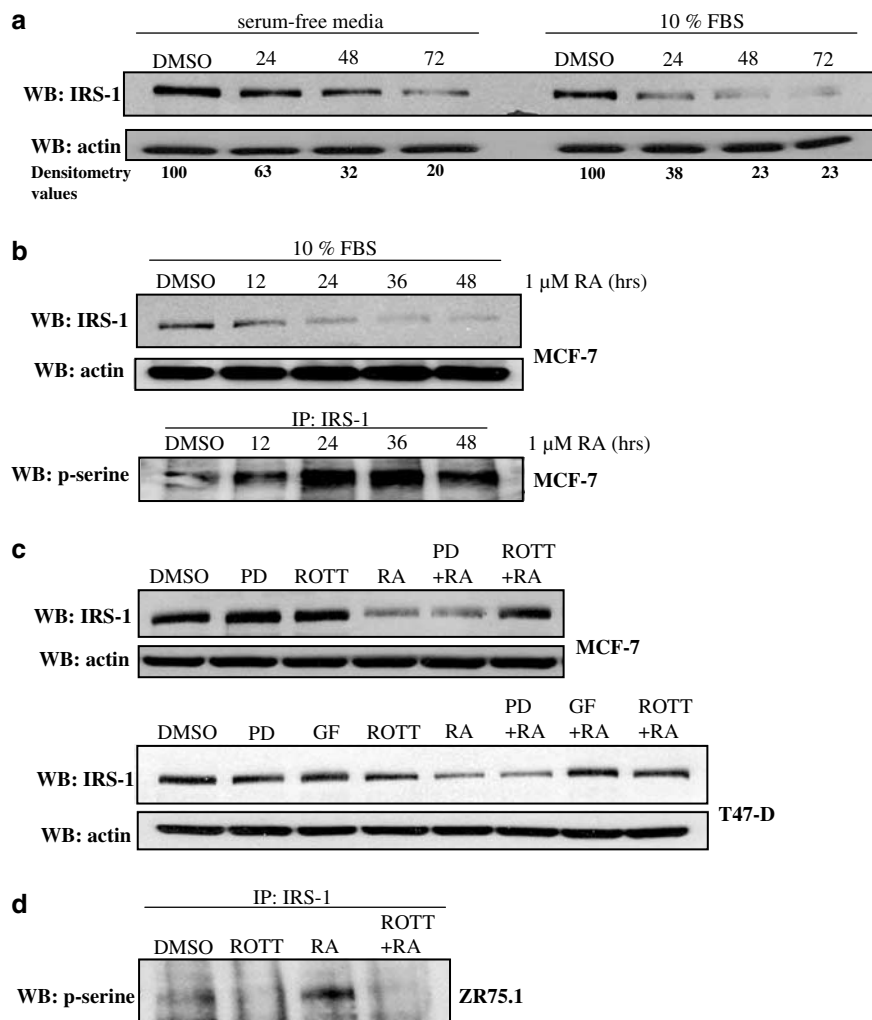


Figure 5 PKC inhibitors block the RA-mediated phosphorylation and degradation of IRS-1. WB was used to determine the expression level of IRS-1 in (a)–(c). (a) MCF-7 cells were treated with 1 μ M RA for 24, 48, and 72 h in SFM or 10% fetal bovine serum (FBS). (b) Top panel: MCF-7 cells were treated with 1 μ M RA for the times indicated in 10% FBS. Lower panel: the total serine phosphorylation status of IRS-1 was determined by immunoprecipitating (IP) IRS-1 from MCF-7 protein lysates and immunoblotting with an anti-phosphoserine (p-serine) antibody. (c) MCF-7 and T47-D cells were pretreated for 60 min with inhibitors of the MAPK (25 μ M PD98059) and PKC (0.5 μ M Rottlerin and 1 μ M GF109203X) signaling cascades and then treated for an additional 48 h with 1 μ M RA in the presence of the inhibitors. (d) ZR75.1 cells were treated as in (c), and the serine phosphorylation status of IRS-1 was determined as in (b)

In vitro phosphorylation of IRS-1 by RA-activated PKC-δ in retinoid-sensitive breast cancer cells

The PKC family of serine/threonine kinases are involved in signaling pathways controlling cell growth, transformation, and differentiation (Blobe *et al.*, 1994). There are at least 10 PKC isoforms, however, Kambhampati *et al.* (2003) recently identified PKC-δ as a selective target of RA. We thus asked whether RA would activate PKC-δ in the breast cancer cell lines used in this study. We found that RA potently induces the phosphorylation of the threonine 505 residue in PKC-δ (Figure 6a). To directly measure if this increase in phosphorylation corresponded to increased activation of PKC-δ, we performed an *in vitro* kinase assays using histone H1 as a

substrate. Figure 6b shows a clear increase in kinase activity with a time course of activation that is consistent with that observed for the RA-mediated decline in IRS-1 protein levels (compare, Figure 6b with Figure 5b). There was no change in PKC-δ activity in the RA-resistant breast cancer cell line MDA-MB-231, suggesting a tight correlation between activation of PKC-δ by RA and regulation of IRS-1 levels (Figure 6c). We confirm that treatment of the cells with 0.5 μM Rottlerin abrogates the RA-induced activity of PKC-δ (Figure 6d, lower panel), consistent with the ability of this inhibitor to rescue the decrease in IRS-1 levels in the RA-sensitive breast cancer cell lines. In addition, we show in Figure 6e that 0.5 μM Rottlerin also blocks basal PKC-δ activity.

Recently Greene *et al.* (2004) identified 18 PKC-δ serine/threonine phosphorylation sites on IRS-1. When three of these sites, serine 307, serine 323, and serine 574, were mutated to alanines, the phosphorylation of IRS-1 in response to activated PKC-δ was significantly decreased. To examine whether activation of PKC-δ by RA increases phosphorylation of IRS-1 on these sites, we performed an *in vitro* kinase assay using the wild-type human IRS-1-GST-fusion protein (GST-IRS-1^{288-678WT}) or the GST-IRS-1²⁸⁸⁻⁶⁷⁸ with serine 307, serine 323, and serine 574 mutated to alanines (GST-IRS-1^{288-678MUT}) as a substrate. Our results show that PKC-δ immunoprecipitated from RA-treated breast cancer cells can phosphorylate the GST-IRS-1^{288-678WT} construct, and that this phosphorylation event is abrogated by coincubation with the PKC-δ inhibitor Rottlerin (Figure 7b, left panel). In addition, when the three putative PKC-δ serine sites are mutated to alanines no RA-induced phosphorylation of the GST-IRS-1^{288-678MUT} construct is observed (Figure 7b, right panel). The possibility that this phosphorylation may result from an interaction between IRS-1 and PKC-δ in the presence of RA is supported by our finding that IRS-1 coimmunoprecipitates with PKC-δ in ZR75.1 cells (and T47-D, data not shown) treated with 1 μM RA (Figure 7c).

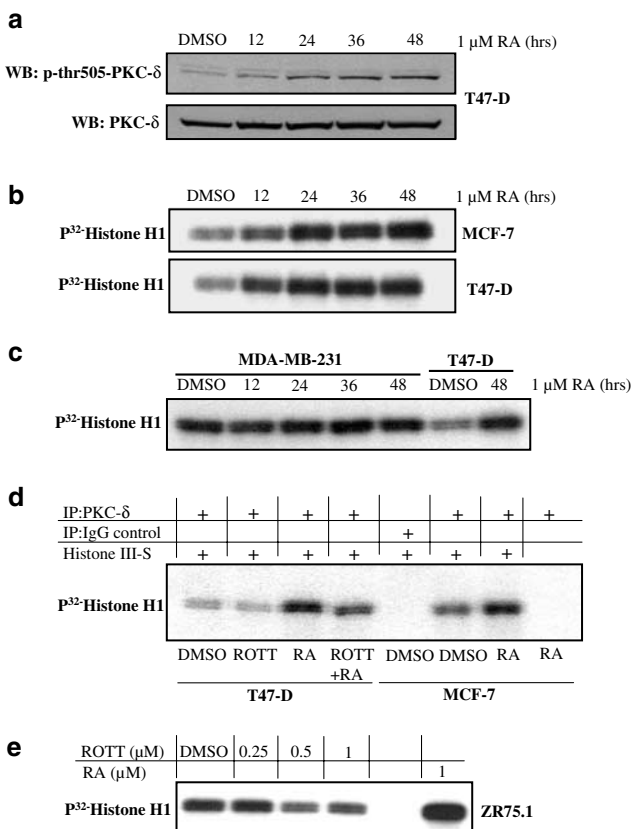


Figure 6 RA activates PKC-δ in retinoid-sensitive breast cancer cells. (a) T47-D cells were treated with 1 μM RA for 12, 24, 36, and 48 h in 10% FBS. Western blot was used to determine the phosphorylation status of anti-PKC-δ at threonine 505 (p-thr505-PKC-δ). This membrane was then stripped and reprobed with an antibody against total PKC-δ. (b) MCF-7 and T47-D cells were treated with 1 μM RA for the time indicated. Lysates were immunoprecipitated (IP) with an anti-PKCδ antibody, and immunoprecipitates were subjected to an *in vitro* kinase assay using histone H1 as an exogenous substrate. Phosphorylated histone H1 was detected by autoradiography. (c) The *in vitro* kinase assay using MDA-MB-231 cell extracts was performed as in (b). (d) T47-D cells were pretreated for 60 min with 0.5 μM Rottlerin and then treated for an additional 36 h with 1 μM RA in the presence of the inhibitor. The *in vitro* kinase assay was performed as in (b). (e) ZR75.1 cells were treated with 0.25, 0.5, or 1 μM Rottlerin for 36 h, and the *in vitro* kinase assay was performed as in (b)

Discussion

Although immediate/early retinoid signaling events are initiated via transcriptional activation of retinoid receptors, later retinoid signaling events can occur through the post-translational regulation of proteins. Considerable attention has been given in recent years to identifying retinoid-regulated proteins to gain insight into the mechanisms involved in their growth inhibitory and differentiating effects (Kim and Lotan, 2004). There is indeed a growing list of proteins known to be regulated by RA via mechanisms involving the ubiquitin-proteasome pathway: cyclin D1 (Spinella *et al.*, 1999), PML/RAR alpha (Yoshida *et al.*, 1996), RAR alpha and RAR gamma (Tanaka *et al.*, 2001), CDK-4 (Sueoka *et al.*, 1999), p300 (Brouillard and Cremisi, 2003), and Skp2 (Dow *et al.*, 2001). Our findings support the addition of IRS-1 to this list. Using breast

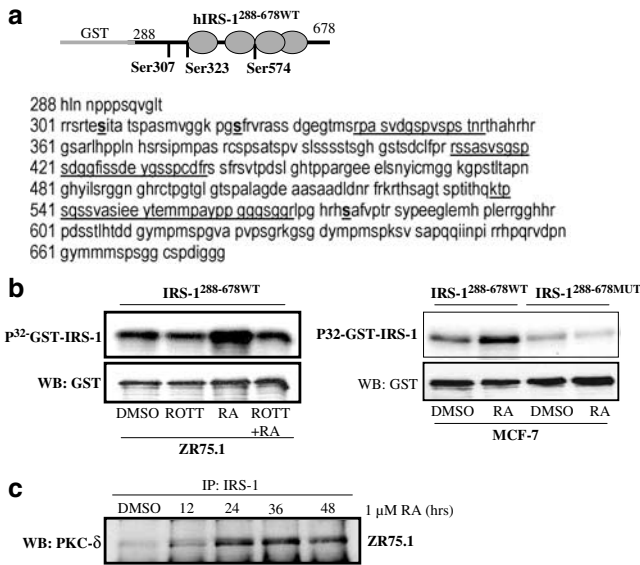


Figure 7 *In vitro* phosphorylation of IRS-1 by RA-activated PKC- δ . (a) Schematic showing the portion of the human IRS-1 protein used in the GST-IRS-1^{288-678WT} construct. Also shown in larger font are the three serine sites phosphorylated in response to activated PKC- δ (serines 307, 323, and 574). The regions underlined in the amino-acid sequence of IRS-1 represent putative PEST domains in the region of the protein from amino acid 288 to 678. The residues in bold font and underlined represent serines 307, 323, and 574. (b) ZR75.1 cells were pretreated for 60 min with 0.5 μ M Rottlerin and then treated for an additional 36 h with 1 μ M RA in the presence of the inhibitor. Lysates were immunoprecipitated (IP) with an anti-PKC- δ antibody, and immunoprecipitates were subjected to an *in vitro* kinase assay using a GST-IRS-1^{288-678WT} or GST-IRS-1^{288-678MUT} (with serines 307, 323, and 574 mutated to alanines) as a substrate. Phosphorylated GST-IRS-1²⁸⁸⁻⁶⁷⁸ was detected by autoradiography. (c) ZR75.1 cells were treated with 1 μ M RA for 12, 24, 36, and 48 h. To detect IRS-1/PKC- δ association, cell lysate was immunoprecipitated with an anti-IRS-1 antibody and immunoblotted with an anti-PKC- δ antibody

cancer cell lines as a model, we are the first to show that RA regulates IRS-1 protein levels through a post-translational mechanism involving the ubiquitin-proteasome pathway. Moreover, we find that PKC inhibitors rescue the RA-mediated loss in IRS-1 protein, and that RA-mediated activation of PKC- δ phosphorylates IRS-1 *in vitro*.

The proteasomal degradation of proteins requires prior binding of ubiquitin to the target protein via three successive reactions. First, the ubiquitin-activating enzyme, E1, activates ubiquitin in an ATP-dependant reaction. Secondly, the ubiquitin molecule is transferred from E1 to the ubiquitin conjugating enzyme, E2. Thirdly, ubiquitin is transferred from E2 to the substrate-specific ubiquitin ligase, E3. In this paper, we show an increased level of IRS-1-ubiquitin conjugates in the presence of RA in MCF-7 cells when proteasomal activity is blocked using MG-132, and confirm this result with cotransfection experiments using flag-IRS-1 and HA-Ub constructs in the three RA-sensitive breast cancer cell lines. We observed that the RA-mediated

increase in the level of IRS-1-Ub conjugates in MCF-7 cells (Figure 4b) is comparable to that previously reported in IGF-I-induced proteasomal degradation of IRS-1 (Lee *et al.*, 2000). Interestingly, we did not observe a decrease in the levels of IRS-2 protein when any breast cancer cell line was treated with RA, suggesting that a specific motif in IRS-1 is responsible for the selective degradation of this protein by RA. It is believed that substrate-specificity of the ubiquitin-proteasome system is due to the specific E3, however, the E3 responsible for the degradation of IRS-1 remains unknown. One can speculate that this E3 may be an SCF ligase complex, since all identified SCF complexes target phosphorylated substrates, and prior studies have shown that IRS-1 needs to be phosphorylated on serine residues prior to its recognition by the ubiquitin machinery (Egawa *et al.*, 2000; Haruta *et al.*, 2000; Greene *et al.*, 2003).

Our observation that RA decreases the levels of IRS-1 more rapidly in the presence of serum than in serum-free media (Figure 6a) suggested that an activated signaling pathway was involved in the degradation of IRS-1. Consistent with the idea that phosphorylated-IRS-1 is targeted for ubiquitination, we show that RA induces total serine phosphorylation of IRS-1. We used a chemical inhibitor approach to begin to clarify the signals generated that target IRS-1 for ubiquitination. We found that PKC inhibitors can rescue the RA-mediated loss in IRS-1 protein in the three RA-sensitive breast cancer cell lines examined. The activation of a number of PKC isoforms, including PKC- δ (Greene *et al.*, 2004), has been linked to serine phosphorylation of IRS-1, and interestingly, Greene *et al.* mentioned an observed decrease in IRS-1 levels when CHO cells were transfected with a constitutively active PKC- δ construct, suggesting the involvement of this kinase in the regulation of IRS-1 protein levels. Our data support such a role of PKC- δ : (1) RA stimulates PKC- δ activity (Figure 6b), (2) RA-activated PKC- δ can phosphorylate IRS-1 *in vitro*, and this is blocked by the PKC- δ inhibitor Rottlerin (Figure 7b, left panel) or by mutation of three critical PKC- δ serine sites in IRS-1 (Figure 7b, right panel), and (3) PKC- δ and IRS-1 interact in the presence of RA (Figure 7c). Consistent with the inability of RA to regulate IRS-1 levels in the RA-resistant MDA-MB-231 breast cancer cell line, we also failed to observe any regulation of PKC- δ in these cells. Although activating PKC has not been previously reported to be required for the ubiquitination of IRS-1, the phosphorylation of p53 by PKC was shown to regulate p53 degradation by stimulating its ubiquitination (Chernov *et al.*, 2001). Interestingly, three of the serine sites (serines 307, 323, and 574) phosphorylated by PKC- δ lie within the C-terminal domain of IRS-1 that contains potential PEST sequences, as identified by a PEST-FIND program (<http://emb1.bcc.univie.ac.at/embnet/tools/bio/PESTfind/>) (Figure 7a). PEST sequences are rich in proline (P), glutamate (E), serine (S) and threonine (T) residues and frequently serve as signals for proteolytic degradation (Rechsteiner and Rogers, 1996). It is tempting to speculate that

PKC- δ -mediated phosphorylation of these three serine sites may induce a conformational change in the C-terminal domain of IRS-1 that enables unmasking of these PEST domains and subsequent recognition by some component of the ubiquitin machinery. We thus propose a model of RA-mediated IRS-1 degradation whereby RA first activates PKC- δ , which in turn phosphorylates IRS-1 on serine residues, allowing for its subsequent recognition by the ubiquitin machinery.

Breast cancer cells exhibit elevated levels of known ubiquitinated proteins, such as cyclin D1 (Wang *et al.*, 1994), cyclin E (Lindahl *et al.*, 2004), and IRS-1 (Rocha *et al.*, 1997). Although the ubiquitin ligase, or E3, for cyclin D1 and IRS-1 remain unknown, future identification of these ligases, may lead to the development of compounds that specifically activate E3, thus inhibiting the accumulation of cyclin D1 and IRS-1 in breast tumors where these proteins are stabilized. A possible novel chemotherapeutic approach for the treatment of breast tumors may be to combine RA with activation of specific E3s to target oncogenic proteins.

Materials and methods

Reagents

All-*trans*-retinoic acid (RA), 9-*cis*-retinoic acid (9*cis*RA), TTNPB, *N*-(4-hydroxyphenyl)retinamide (4-HPR), and 17- β -estradiol (E2) were purchased from Sigma. Bexarotene was a generous gift of Ligand Pharmaceuticals, Inc. (San Diego, CA, USA). Recombinant human IGF-I was purchased from PeptoTech (Princeton, NJ, USA). MG-132 (MG), 1,25-dihydroxyvitamin D3, 15-deoxy- δ 12,14-prostaglandin J2, Rottlerin and GF109203X were purchased from Calbiochem. Protein G-agarose, and Nonidet-P40 (NP-40) were purchased from Sigma (Oakville, Canada). Enhanced chemiluminescence (ECL) detection system and Protein A-sepharose were purchased from Amersham Biosciences. The following antibodies (Abs) were used for immunoprecipitations: anti-C-terminal IRS-1 pAb (Upstate Biotechnology), anti-FLAG pAb (Sigma), anti-PKC- δ pAb (Santa Cruz Biotechnology). Serine phosphorylation was detected with an anti-phosphoserine pAb (Zymed). The following antibodies were used for Western blotting: anti-IRS-1 pAb, anti-ubiquitin mAb (Santa Cruz Biotechnology), anti-HA-peroxidase mAb (clone 12CA5, Roche), anti-phospho-IRS-1 (Ser³⁰⁷) pAb (Upstate Biotechnology), anti-GRB2 mAb (Transduction laboratories), anti-c-Jun pAb (Santa Cruz), anti-phospho-PKC- δ (Thr⁵⁰⁵) pAb (Cell Signaling), anti-PKC- δ pAb (Santa Cruz), and anti-GST mAb (Santa Cruz). Equal loading in Western blotting experiments was assessed using either an anti-lamin B pAb (Santa Cruz) or anti- β -actin mAb (Sigma).

Cell maintenance and total cell lysate preparation

MCF-7 cells, ZR75.1 cells, and MDA-MB-468 cells were maintained in phenol red containing α -MEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS). T47-D cells and MDA-MB-231 cells were maintained in phenol red containing DMEM/F-12 (Life Technologies, Inc) supplemented with 10% FBS. When experiments were performed under serum-free conditions, cells at 70% confluence were washed twice with phosphate-buffered saline and

changed to phenol red-free α -MEM supplemented with BSA and holo-transferrin (serum-free media – SFM). When experiments were performed in the presence of serum, cells were treated in the same media in which they were routinely maintained. For total cell lysate preparation, all cell lines were washed twice with cold phosphate-buffered saline (PBS) and lysed with RIPA buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP-40, 0.05% sodium deoxycholate, 0.1% SDS) containing protease and phosphatase inhibitors. Subsequent to incubation of lysate on ice for 30 min, the extracts were centrifuged at 13 000 *g* at 4°C for 30 min to remove insoluble material. After centrifugation, the protein content was measured by the Bradford assay using Bio-Rad reagents and BSA as standard.

Subcellular fractionation

Following the treatment periods in DMSO or RA, MCF-7 cells were washed in PBS and incubated on ice for 10 min in buffer 1 (10 mM Tris-Cl pH 7.8, 10 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 300 mM sucrose, 0.5 mM DTT, plus protease and phosphatase inhibitors). A volume of 5 μ l of 10% NP-40 was added to each sample and vortexed to lyse cells. The samples were centrifuged for 1 min at \sim 1000 r.p.m., and the supernatant collected (cytosolic fraction). The pellet was washed 3 times with washing buffer (50 mM NaCl, 10 mM HEPES, pH 8, 25% glycerol, 0.1 mM EDTA, 100 mM DTT, plus protease and phosphatase inhibitors), and resuspended in buffer 2 (20 mM Tris-Cl pH 7.8, 5 mM MgCl₂, 320 mM KCl, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, plus protease and phosphatase inhibitors). Tubes were incubated on ice for 30 min followed by centrifugation for 15 min at 13 000 *g*; the supernatant represented the nuclear fraction. A measure of 20 μ g of cytosolic and nuclear proteins were then subjected to SDS-PAGE and transferred to nitrocellulose membrane filters. The membranes were processed for Western blotting of IRS-1, GRB-2 (for cytoplasmic purity), c-Jun (for nuclear purity), and actin (loading control).

Immunoprecipitation and Western blotting

For Western blotting, cell lysates were boiled for 3 min in 2 \times SDS sample buffer (250 mM Tris-HCl pH 6.8, 8% SDS, 8 mM EDTA, 35% glycerol, 2.5% β -mercapto-ethanol, bromophenol blue) and resolved in 8–10% SDS-polyacrylamide gels (SDS-PAGE). For immunoprecipitation, 500 μ g–1 mg of precleared cell lysates were incubated with the indicated antibody overnight at 4°C, followed by the addition of protein G-agarose overnight to collect immune complexes. The immunoprecipitates were washed in RIPA buffer, resuspended in SDS sample buffer, and boiled for 5 min. The solubilized proteins were resolved by SDS-PAGE. Proteins on the gel were transferred to nitrocellulose membrane (Bio-Rad) and detected by Western blotting with the indicated antibody using ECL. Some membranes were stripped to prepare them for a second round of probing.

Ubiquitination of IRS-1

The ubiquitination of IRS-1 was examined by transfecting (using FuGENE, Roche) breast cancer cells with a FLAG-tagged human IRS-1 cDNA in an expression construct provided by Dr Chris Sell, and a hemagglutinin (HA)-tagged ubiquitin cDNA in an expression construct provided by Dr Dirk Bohmann (Treier *et al.*, 1994). At 24 h following transfection, cultures were washed twice with PBS and incubated in media containing 10 μ M RA for 24 h in the presence or absence of 10 μ M MG132 for the last 12 h of RA

treatment. FLAG-tagged IRS-1 protein was immunoprecipitated using an anti-FLAG antibody (Sigma) and after separation of the immunoprecipitated proteins by SDS-PAGE, the ubiquitinated IRS-1 was detected using an anti-HA-Peroxidase mAb (clone 12CA5, Roche).

Northern blotting

Total cellular RNA was isolated using guanidinium thiocyanate extraction as previously described (Chomczynski and Sacchi, 1987). For Northern blotting, 20 μ g of RNA was electrophoresed on a 1% formaldehyde agarose gel and blotted onto Zeta probe (BioRad, Mississauga, Ontario, Canada) transfer membranes. cDNA probes were labeled by random priming (Amersham Biosciences). Hybridization and autoradiography was performed as previously described (Rosenauer *et al.*, 1996). The full-length cDNA encoding IRS-1 was the generous gift of Dr Khan (Joslin Diabetes Center).

RT-PCR

Total cellular RNA was isolated using guanidinium thiocyanate extraction as previously described (Chomczynski and Sacchi, 1987). The methods described by Morelli *et al.* (2003) were used for IRS-1 RT-PCR. In brief, RNA (1 μ g) was reverse transcribed (Superscript First Strand synthesis system – Gibco) and then amplified by PCR to obtain products corresponding to cDNA fragments of IRS-1. The following primers were used: IRS-1 upstream primer 5'-TCCACTGTGACACCAGAATAAT-3' and IRS-1 downstream primer 5'-CGCCAACATTGTTTCATTCCAA-3'. The following PCR conditions were used for IRS-1: 1 min at 94°C, 1 min at 50°C, 2 min at 72°C. The amplification products obtained in 30 cycles were analysed in a 1% agarose gel.

Pulse analysis

To determine the synthesis rate of IRS-1, we followed the methods described by Zhang *et al.* with some modifications (Zhang *et al.*, 2000). At 24 h after plating, MCF-7 cells were treated with DMSO vehicle or 10 μ M RA for 24 h. Prior to labeling, the medium was replaced with methionine-free RPMI media (Wisent Inc.) for 1 h. During the last 12 h of RA treatment, the cells were labeled with 100 μ Ci of [³⁵S]methionine (Pro-mix, Amersham Biosciences) and cells were collected after 0, 4, 8, and 12-h time intervals. Total cellular proteins were isolated and IRS-1 immunoprecipitated (as described above). The IRS-1 immunoprecipitates were subjected to SDS-PAGE, and following transfer to nitrocellulose filters, labeled IRS-1 was visualized by autoradiography.

Pulse-chase analysis

To determine the degradation rate of IRS-1, we followed the methods described by Zhang *et al.* with some modifications (Zhang *et al.*, 2000). At 24 h after plating, MCF-7 cells were treated with either DMSO vehicle or 10 μ M RA for 24 h. Prior to labeling, the media was replaced with methionine-free RPMI media (Wisent Inc.) for 1 h. The cells were then pulsed for 4 h with 100 μ Ci of [³⁵S]methionine (Pro-mix, Amersham Biosciences). Following the pulse, the cells were chased for 0, 4, 8, and 12 h in 10% FBS containing media. After these chase times, total cellular proteins were isolated and processed as described above for the pulse analysis experiments.

Immunofluorescence

One day after plating on coverslips, MCF-7 cells were treated with DMSO vehicle or 10 μ M RA for 48 h. For the last 12 h of RA treatment, 10 μ M MG-132 was added. Following the treatment period, the cells were fixed in 3% formaldehyde in PBS for 30 min. Next, the cells were permeabilized in PBS containing 0.2% Triton X-100 for 5 min. The cells were then incubated for 3 h with a rabbit anti-IRS-1 antibody (2 μ g/ml), washed in PBS, and incubated for 1 h with a rhodamine-conjugated donkey anti-rabbit IgG secondary antibody. Following additional washes in PBS, the coverslips were incubated with 4,6-diamidino-2-phenylindole (DAPI) for 5 min and mounted on glass slides in antifade medium. The images were then collected using a fluorescence microscope.

In vitro kinase assay using histone H1

Cells were treated with RA or Rottlerin for the indicated times and then lysed in phosphorylation lysis buffer A (20 mM Tris-Cl pH 7.5, 150 mM NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, 1% NP-40, 1 mM sodium orthovanadate, 10 mM NaF, 2 mM sodium pyrophosphate, 1 mM PMSF). Cell lysates were immunoprecipitated with anti-PKC- δ antibody, followed by the addition of protein A-sepharose overnight to collect immune complexes. Immunoprecipitates were washed twice with lysis buffer A and twice with wash buffer B (25 mM Tris-Cl pH 7.5, 5 mM MgCl₂). Protein A-sepharose beads were resuspended in 30 μ l of kinase buffer (25 mM Tris-Cl pH 7.5, 5 mM MgCl₂, 0.5 mM EDTA, 1 mM dithiothreitol, 20 μ g of phosphatidylserine, and 20 μ M ATP) containing 5 μ g of histone H1 (Sigma Type III) as an exogenous substrate, and [γ -³²P]ATP. The reaction was incubated for 30 min at room temperature and terminated by the addition of SDS sample buffer. Proteins were separated by SDS-PAGE, and phosphorylated histone H1 was detected by autoradiography.

In vitro kinase assay using GST constructs

Cells and cell extracts were treated as described above (*In vitro* kinase assay using histone H1), however, in the kinase reaction, GST-IRS-1^{288–678WT}, GST-IRS-1^{288–678MUT}, or GST alone was used as an exogenous substrate. The GST constructs were a generous gift from Dr R Roth, and have been previously described in Greene *et al.* (2004). GST-IRS-1^{288–678WT} or GST were then purified from the reaction mix with GSH-Sepharose beads, resolved by SDS-PAGE, and phosphorylated GST-IRS-1^{288–678WT} was detected by autoradiography. Decayed membranes were then incubated with an anti-GST antibody to ensure equal pull down of GST.

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