Mutation of Di-Leucine Residues in the Juxtamembrane Region Alters EGF Receptor Expression†

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ABSTRACT: Di-leucine motifs have been implicated in the internalization or degradation of many membrane proteins. The epidermal growth factor receptor (EGFR) contains two di-leucine residues at 658 (TLRRLLQER) and 679 (NQALLRIL). To determine the role of these di-leucine motifs in regulating EGF receptor expression, activity, or ligand-induced degradation, the di-leucine residues at positions 658 or 679 were mutated to di-alanine residues, and the mutant receptors were stably expressed in CHO cells. The results indicate that mutation of either di-leucine motif generates and promotes cell surface expression of carboxy-truncated EGF receptors (M_r 120, 140 kDa) that do not undergo EGF-induced autophosphorylation or degradation. In contrast, full-length EGF receptors (170 kDa) containing di-alanine substitutions resemble wild type receptors in that they respond to EGF by autophosphorylation, their tyrosine kinase activity is inhibited by protein kinase C, and they are degraded. The level of autophosphorylation of the 170 kDa mutant receptors and EGF-induced tyrosine phosphorylation of other cellular proteins is lower than that of the wild type receptor, consistent with formation of kinase-inactive heterodimers between the truncated and full-length mutant receptors. These results demonstrate that removal of either of the di-leucines leads to generation of inactivating carboxy-truncated receptors, suggesting that the two di-leucine motifs within the juxtamembrane region of the EGFR are important for ensuring normal receptor expression.

Cellular growth is mediated through the expression of discrete cell surface receptors such as epidermal growth factor receptors (EGFRs)[1] that respond to growth factors by activation of a kinase signaling cascade. Truncation of these receptors to remove negative regulatory domains can lead to deregulated kinase activity, resulting in uncontrolled growth and tumorigenesis (Wells et al., 1990). Alternatively, loss of kinase activity by mutation of the ATP binding site or truncation within the kinase domain leads to formation of kinase-inactive receptors that can dimerize with wild type EGFR and act as dominant-negative suppressors of wild type receptor signaling (Kashles et al., 1991). Thus, maintaining the fidelity of growth factor receptors is important for proper growth control.

In order to ensure that signals triggered by growth factors are not prolonged indefinitely, cells have developed mechanisms for rapid removal of activated receptors from the cell surface. For example, previous studies with truncated EGFR mutants have shown that residues within the internal juxtamembrane domain (residues 647−958) are responsible for lysosomal targeting and degradation of the receptor, and these regions are distinct from those regulating internalization (Herbst et al., 1994). Di-leucine motifs have been implicated in the lysosomal targeting, Golgi sorting, and internalization of a number of membrane proteins (Aiken et al., 1994; Chen et al., 1993; Cleghon & Morrison, 1994; Letourneur & Klausner, 1992; Oda et al., 1993; Ogata & Fukuda, 1994). Interestingly, the juxtamembrane region of the EGFR possesses two closely apposed di-leucine repeats at 658 and 679.

To determine whether either of these di-leucine motifs plays a role in regulating the expression or degradation of the EGFR, we mutated the di-leucine sequences to di-alanines and stably expressed the mutant receptors in CHO cells. Previous studies have demonstrated that substituting di-alanine for di-leucine, a relatively conservative change, is sufficient to block internalization and lysosomal targeting of several membrane proteins (Dietrich et al., 1994; Letourneur & Klausner, 1992; Pieters et al., 1993; Pond et al., 1995). The results of the present study suggest that loss of the juxtamembrane di-leucine residues leads to the generation and cell surface expression of aberrant, truncated EGF receptors.

EXPERIMENTAL PROCEDURES

Generation of Cell Lines Expressing EGF Receptor Mutants. CHO cells, maintained in Ham's F-12 media supplemented with 10% fetal bovine serum (FBS), were stably transfected by the $Ca_3(PO_4)_2$ method as previously

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[1] Abbreviations: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; MAP, mitogen-activated protein; PDBu, phorbol 12,13-dibutyrate; CHO, Chinese hamster ovary cells; di-Ala, di-alanine; di-Leu, di-leucine; CMV, cytomegalovirus; PKC, protein kinase C; IL-6, interleukin 6; PBS, phosphate-buffered saline; TBST, Tris-buffered saline−0.2% Tween-20; PMSF, phenylmethanesulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; KIU, kallikrein-inactivating unit.

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described (Morrison et al., 1993; Sambrook et al., 1989), either with a wild type EGF receptor construct (11WT) or with the following EGFR mutants: (1) alanine substituted for leucine at 658 and 659 (di-Ala658), (2) alanine substituted for leucine at 679 and 680 (di-Ala679), (3) both together (di-Ala658,679). The constructs were cloned into a pCB6$^+$/ER vector containing a cytomegalovirus (CMV) promoter linked to the receptor cDNA and a neomycin resistance gene. Cells were selected in 200 μg/mL G418, and a fluorescence-activated cell sorter (FACS) was used to select populations with similar receptor expression that were characterized in the studies described in this paper. For this purpose, the R1 EGFR antibody (α-EGFR) (Waterfield et al., 1982) was used in conjunction with a fluorescein-conjugated a-mouse IgG (Sigma). 2WT is a clonal cell line derived by expressing wild type EGFR in CHO cells and has been described previously (Morrison et al., 1993).

Immunoblotting of EGF Receptor Protein. Equivalent protein aliquots from whole cell protein extracts in RIPA buffer (20 mM HEPES, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate, 5 mM EDTA, 50 mM NaCl, 25 mM sodium pyrophosphate, 5 mM NaF, 50 μM Na_3VO_4, 200 KIU/mL aprotinin, 5 μM leupeptin, and 1 mM PMSF, pH 8.0) isolated from cells expressing either di-Ala658, di-Ala679 or wild type receptors were resolved by 6−7% SDS−PAGE under reducing conditions with a Fisher semidry transfer apparatus according to the Fisher protocol. The nitrocellulose was blocked with 5% bovine serum albumin (BSA) in 50 mM Tris, pH 7.5, 150 mM NaCl, and 0.2% Tween-20 (TBST), and the blots were then probed with primary antibodies in TBST. For EGFR analysis, samples were probed with mouse monoclonal antisera (11E8) raised against a trpE fusion protein containing 330 amino acids of the cytoplasmic v-*erbB* domain (Tucker et al., 1993), or with rabbit monoclonal anti-EGFR antisera (1005) (Santa Cruz Biotechnology, Inc). The EGFR band was detected by secondary antibodies conjugated to horseradish peroxidase (HRP), visualized by Renaissance chemiluminescence (New England Nuclear), and analyzed by densitometric scanning using an Ambis Radioanalytic Imaging System.

EGFR and Cellular Protein Tyrosine Phosphorylation. Equivalent protein aliquots from whole cell protein extracts in RIPA buffer were isolated from cells expressing CHO 2WT, 11WT, di-Ala658, or di-Ala679. Protein was resolved by SDS−PAGE. The gels were immunoblotted, probed with mouse monoclonal anti-phosphotyrosine antibodies, and analyzed as described above. IgG2b$_k$ (Upstate Biotechnology Inc.) was used to detect cellular tyrosine phosphorylation, and PY20 (Transduction Laboratories) was used to detect EGFR autophosphorylation.

Metabolic Analysis. Confluent cells were incubated in DMEM lacking methionine or methionine and cysteine (Gibco) for 4 h. Cells were labeled with 100 μCi/mL Tran^{35}S-label™ (ICN) for 30 min or 70 μCi/mL ^{35}S-Expre^{35}S^{35}S (New England Nuclear) for 30 min. Cells were harvested immediately or were chased for 6 h with 10× methionine in Ham's F-12 (Whittaker). Cells were lysed in RIPA buffer and immunoprecipitated with precoupled α-EGFR R1 antibody bound to protein A Sepharose, and the eluted protein was resolved by SDS−PAGE. Gels were soaked in EN^{3}HANCE (Dupont) and visualized by autoradiography.

Cell-Surface Receptor Analysis. Plates of confluent serum-starved cells were placed on ice, washed with PBS, and incubated at 4 °C with 0.1 mg/mL sulfo-NHS-biotin (Pierce) in PBS-CM (0.1 mM $CaCl_2$, 1.0 mM $MgCl_2$) for 20 min. For cell surface receptor labeling, free reactive biotin was quenched by washing twice with 50 mM glycine. For analysis of cell surface receptor degradation, free reactive biotin was quenched with 50 mM glycine, and cells were washed with Ham's F-12 prior to treatment with EGF as described in the figure legends. Cells were then lysed in RIPA and immunoprecipitated with precoupled α-EGFR R1 antibody bound to protein A Sepharose. The proteins were resolved by SDS−PAGE, detected by probing with streptavidin-HRP (Sigma), visualized by Renaissance chemiluminescence (New England Nuclear), and analyzed as described above.

EGF Internalization. Plates of confluent serum-starved cells were placed on ice, washed with PBS, and incubated for 2 h at 4 °C with 0.4 nM ^{125}I-EGF (New England Nuclear) in chilled EGF binding buffer (DMEM with 50 mM HEPES, 0.1% BSA, pH 7.4). Cells were washed extensively with PBS to remove unbound ligand. To measure receptor internalization, Ham's F-12 was added to cells and they were then incubated at 37 °C for various times. The media was aspirated off, and cell-stripping buffer (50 mM glycine hydrochloride, 100 mM NaCl, 2 mg/mL poly(vinylpyrrolidone), 2 M urea, pH 3.0) was added for 10 min on ice to remove surface-bound ligand. The cells were then lysed in 1 N NaOH, and this extract constituted the internalized EGF pool. The radioactivity was quantitated by an LKB γ counter.

RESULTS

Expression of Mutant Di-Ala EGFRs. To investigate whether the two membrane proximal di-leucines in the EGFR play a role in regulating receptor expression or degradation, the di-leucine residues at positions 658 or 679 were mutated to di-alanine residues either singly (di-Ala658 or di-Ala679) or as a double mutation (di-Ala658,679). The mutagenized EGFR cDNAs, linked to a CMV promoter in a vector expressing the neomycin resistance gene, were transfected into CHO cells. Stable transfectants were selected by resistance to G418, and the drug-resistant cells were further selected for equivalent surface EGF receptor expression by FACS sorting using an antibody (α-EGFR R1) to an external EGFR epitope. Populations of cells expressing similar cell surface levels of either wild type (11WT) or singly mutated di-leucine residues (di-Ala679 or di-Ala658) were then characterized. Although cells expressing either of the single di-alanine mutant EGFR receptors were readily isolated by FACS analysis, no double mutants were detected by either antibody binding to cell surface receptors or immunoblot analysis (data not shown). The inability to isolate a cell line stably expressing the di-Ala658,679 EGFR suggests that the doubly mutated receptor may be destabilized and degraded upon alteration of both di-leucine sequences.

Cell Surface Expression of Di-Ala Mutant EGFRs. The use of FACS to isolate cells expressing the mutant receptors indicated that one or more of the mutant receptors were expressed on the cell surface. To characterize the species transported to the cell surface, the extracellular domains of the di-Ala658 or di-Ala679 mutant EGFRs were labeled with

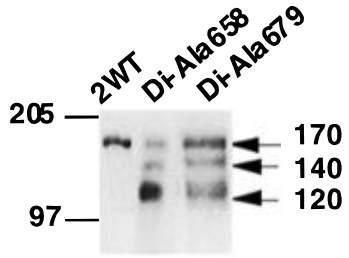

FIGURE 1: Cell surface biotinylation of EGFR protein species. Equivalent numbers of CHO cells expressing wild type (2WT, lane 1), Di-Ala658 (lane 2), and Di-Ala679 (lane 3) mutated EGFRs were surface biotinylated. EGFRs were immunoprecipitated, and the biotinylated receptors were analyzed as described in Experimental Procedures. The data are representative of 3 independent experiments.

reactive biotin (sulfo-NHS-biotin). The receptors were immunoprecipitated with α-EGFR R1 antibody from surface-labeled cells, and the immunoprecipitates were resolved by SDS−PAGE and transferred to nitrocellulose membranes. The membranes were probed with streptavidin conjugated to horseradish peroxidase, and the expression pattern of the biotinylated receptors was assessed. SDS−PAGE analysis revealed three EGFR species from cells expressing either the di-Ala658 or di-Ala679 EGFRs (Figure 1). The largest mutant receptor comigrated with the wild type receptor (170 kDa) while the two smaller species migrated with apparent molecular weights of 140 and 120 kDa. The relative proportion of the three receptors differed between the two mutants. In cells transfected with the di-Ala679, almost 50% of the expressed receptor was full-length, whereas in cells expressing the di-Ala658 mutant, the 120 kDa receptor was the predominant form.

Relationship between the Full-Length and Truncated Di-Ala658 Mutant EGFRs. Cells expressing di-Ala658, the mutant that generated the largest proportion of the truncated species, were metabolically labeled with [^{35}S]methionine/cysteine prior to immunoprecipitation with α-EGFR R1 antibody. The results revealed three metabolically labeled di-Ala658 EGF receptors that were similar in size (120, 140, 170 kDa) to those expressed at the cell surface (Figure 2). Furthermore, the ratios of the three species were similar to those observed by cell surface labeling.

In order to determine if there is a precursor−product relationship between the full-length and truncated receptors, pulse chase analysis was performed. Cells expressing the di-Ala658 mutant receptors were labeled with [^{35}S]methionine/cysteine for 30 min followed by a 6 h chase with nonradioactive methionine. There was no change in the relative intensities of the 3 bands following the chase, although a slight retardation of mobility is evident (Figure 2A), suggesting that all three species are stable and undergo maturation. These results indicate that the three receptors are present soon after synthesis, possibly as a result of premature termination or proteolysis. EGF receptors of similar size can also be detected by metabolically labeling CHO cells expressing wild type receptor with [^{35}S]methionine/cysteine (Figure 2B). However, in the case of the wild type receptors, the proportion of full-length receptor is much larger.

Identification of Multiple Receptor Species. To characterize the mutant EGF receptors further, two other α-EGFR antibodies were used for immunoblot analysis of whole cell

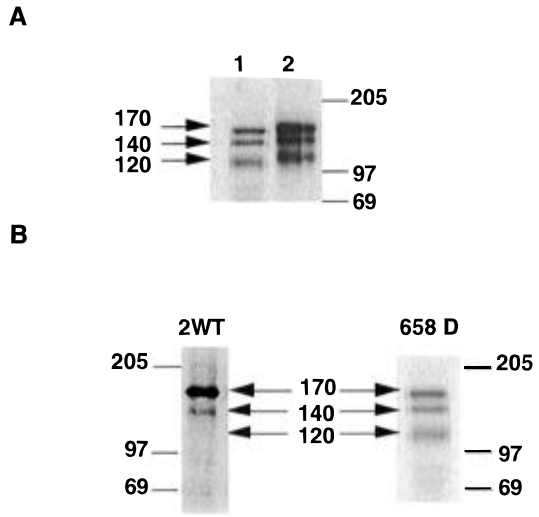

FIGURE 2: Metabolic labeling of di-Ala658 EGFR. (A) Autoradiogram depicting the pulse−chase analysis of di-Ala658 EGFR. CHO cells expressing di-Ala658 EGFR were metabolically labeled with [^{35}S]methionine/cysteine. The cells were either harvested immediately upon labeling for 30 min with 100 μCi/mL [^{35}S]-methionine/cysteine (lane 1) or chased for an additional 6 h (lane 2). The cells were lysed and immunoprecipitated as described in Experimental Procedures. The data are representative of 3 independent experiments. (B) Autoradiogram depicting the metabolic labeling of di-Ala658 or wild type EGFRs expressed in CHO cells. Cells expressing di-Ala658 EGFR were labeled for 30 min with 100 μCi/mL [^{35}S]methionine/cysteine while those expressing wild type EGFR (2WT) were labeled for 30 min with 70 μCi/mL [^{35}S]-methionine/cysteine. The EGFR was immunoprecipitated and analyzed as described in Experimental Procedures. The data are representative of 2 independent experiments.

extracts from stably transfected cells expressing either the di-Ala658 or di-Ala679 receptors. Initially, proteins were blotted with a mouse monoclonal antibody (11E8) raised against a trpE fusion protein containing 330 amino acids of the cytoplasmic v-*erbB* domain. This antibody recognized two species in both mutants: the 170 kDa species that comigrated with the wild type EGFR, and the 140 kDa species (Figure 3A,B). Thus, it appears that the smallest 120 kDa species has the extracellular domain, detected by cell surface labeling as well as the α-EGFR R1 antibody, but is truncated at the carboxy terminus and lacks the 11E8 cytoplasmic domain epitope. These data, in conjunction with the molecular weight, suggest that the 120 kDa EGF receptor has a carboxy-terminal deletion that extends to or near the ATP binding site (around residue 721) within the kinase domain (Livneh et al., 1986).

Further analysis with a different α-EGFR antibody (1005), directed against the peptide from residues 1005−1016 in the carboxy terminus of the EGFR, detected only the largest 170 kDa receptor species for both di-Ala mutants and the wild type EGFR (Figure 3C). This result, together with the cell surface labeling and the 11E8 antibody crossreactivity, suggests that the 140 kDa species is a carboxy-truncated EGF receptor that lacks the 1005−1016 epitope recognized by this antibody. The 1005−1016 residues are located within the CAIN domain that codes for an internalization signal (Chen et al., 1989; Nesterov et al., 1995).

Mutant Receptors Have Reduced Levels of Autophosphorylation and Undergo Normal Down Regulation by PKC. Upon EGF stimulation of cells expressing the mutant di-Ala658 or di-Ala679 receptors, only the 170 kDa receptor was autophosphorylated at tyrosine residues (Figure 4A).

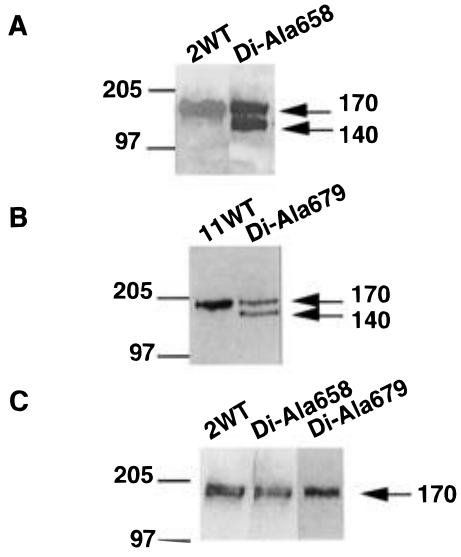

FIGURE 3: Immunoblot analysis of wild type and di-Ala mutant EGFRs. Cells were plated at equivalent numbers and serum-starved overnight, and extracts were prepared for immunoblot analysis as described in Experimental Procedures. (A) Extracts from cells expressing wild type (2WT) or di-Ala658 EGFR were probed with α-EGFR 11E8. (B) Extracts from cells expressing wild type (11WT) or di-Ala679 EGFR were probed with α-EGFR 11E8. (C) Extracts from cells expressing wild type (2WT), or di-Ala658 or di-Ala679 EGFRs were probed with α-EGFR (1005). The data are representative of 4 independent experiments.

However, the extent of 170 kDa mutant receptor autophosphorylation, when normalized to the amount of 170 kDa receptor determined by immunoblotting, was lower than that of the wild type receptor (Figure 4B). For both mutants, the extent of the decrease in autophosphorylation reflected the relative proportion of 120 kDa truncated receptors. Formation of kinase-inactive heterodimers between the 170 and 120 kDa receptors is one mechanism that could account for the reduced autophosphorylation observed for both mutants.

Neither the 120 kDa nor the 140 kDa di-alanine mutant receptors were autophosphorylated in response to EGF. This result is not surprising, since both truncated receptors have lost most or all of the tyrosine phosphorylation sites, and the 120 kDa receptor has additionally lost most if not all of the tyrosine kinase domain and is probably kinase-inactive. In cells expressing the di-Ala658 receptor, which is largely in the truncated form, there was a dramatic reduction in EGF-induced receptor autophosphorylation. To determine whether the reduction in autophosphorylation corresponded to a reduction in EGFR tyrosine kinase activity or EGF signaling, we measured the EGF-induced tyrosine phosphorylation of cellular proteins (Figure 4C). The observed reduction in EGF-induced tyrosine phosphorylation suggests that the expression of these carboxy-truncated receptors inhibits EGFR signaling.

In order to assess whether the mutant receptor kinase activity is regulated like that of the wild type EGFR, sensitivity to phorbol ester down regulation was measured by EGF-induced autophosphorylation. The di-Ala658 and 679 mutant receptors exhibited a dose-dependent decrease in autophosphorylating activity in response to phorbol dibutyrate (PDBu) treatment, similar to that observed in the wild type receptor (Figure 5). This result suggests that the mutant receptor kinase activity is regulated normally by PKC.

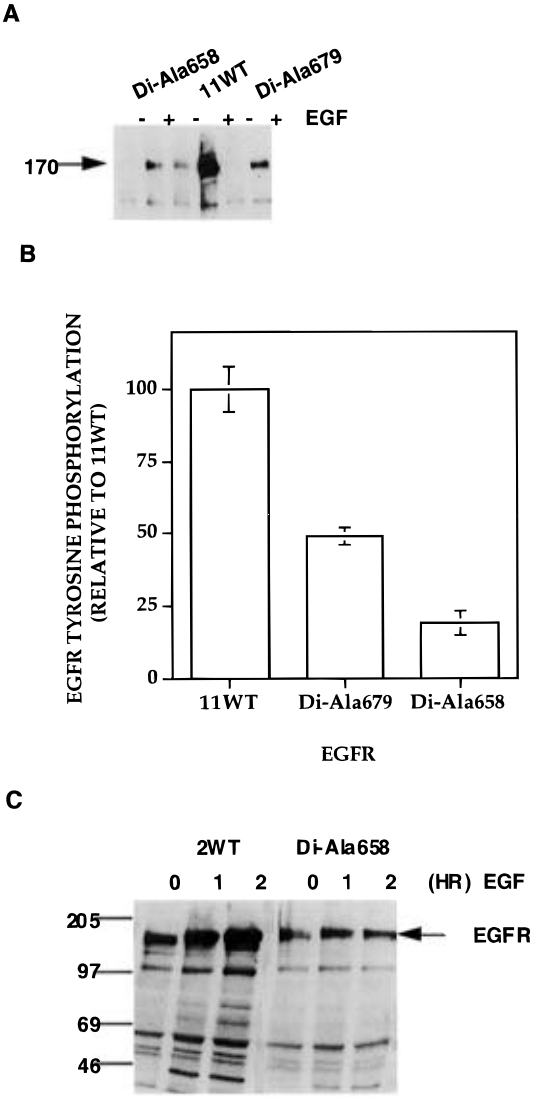

FIGURE 4: Di-Ala EGFRs have reduced EGF-induced autophosphorylation and tyrosine kinase activity. Equivalent numbers of CHO cells expressing the wild type or mutant EGF receptors were plated, serum-starved and treated as described below. Autophosphorylation and tyrosine phosphorylation of total cellular proteins of wild type and mutant EGFR expressing cells were measured by immunoblotting with anti-phosphotyrosine antibodies as described in Experimental Procedures. (A) Immunoblot analysis demonstrating EGF-induced autophosphorylation in cells expressing di-Ala679 (lanes 1, 2), wild type (11WT, lane 3, 4), or di-Ala658 (lanes 5, 6) EGFRs. Cells were treated without (lanes 1, 3, and 5) or with (lanes 2, 4, and 6) EGF. (B) Densitometric quantitation of relative EGFR tyrosine phosphorylation levels of wild type and both di-Ala EGFRs. Receptor levels were determined by immunoblotting with 11E8 α-EGFR, and the relative EGFR tyrosine phosphorylation was determined by dividing the level of EGFR phosphotyrosine (using PY20) by the level of expression of the full-length receptor (using 11E8). Data are shown as % of wild type (11WT) EGFR tyrosine phosphorylation. Error bars represent deviation from the mean of duplicate samples. (C) Immunoblot analysis demonstrating EGF-induced tyrosine phosphorylation in cells expressing wild type (2WT, lanes 1−3) or di-Ala658 (lanes 4−6) EGFRs. Serum-starved cells were treated with or without 10 nM EGF for indicated times and prepared as described in Experimental Procedures (using $IgG2b_k$). The data are representative of at least 2 independent experiments.

Cells Expressing the Mutant Receptors Have Reduced Ligand Internalization Rates. To determine whether loss of either di-leucine motif impaired ligand-induced internalization, internalization of surface-bound EGF was assessed. EGF

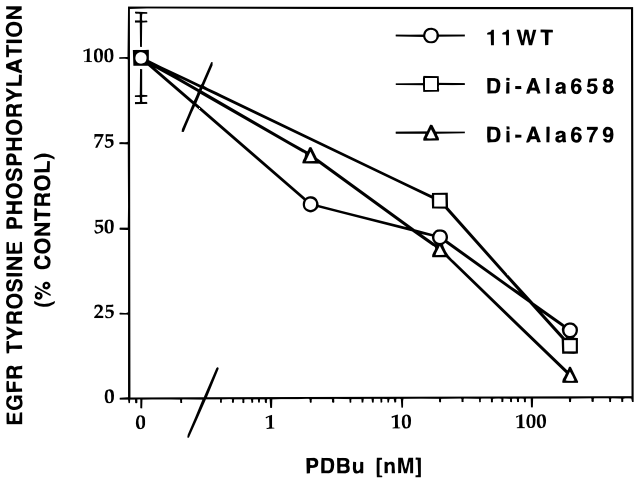

FIGURE 5: Di-Ala EGFR Autophosphorylation Is Inhibited by Protein Kinase C. Equivalently plated cells were serum-starved and treated for 10 min without or with increasing doses of PDBu. Then 10 nM EGF was added to the cells for 4 min prior to lysing the cells in RIPA buffer. Immunoblot analysis with anti-phosphotyrosine antibody of di-Ala658 (□) and di-Ala679 (△) mutated EGFRs, or the wild type (○, 11WT) EGFRs, was performed as described in Experimental Procedures. EGFR autophosphorylation, quantitated by densitometry, is plotted relative to control samples treated with EGF alone. Error bars represent deviation from the mean of duplicate samples treated with EGF alone. The data shown are representative of 2 independent experiments.

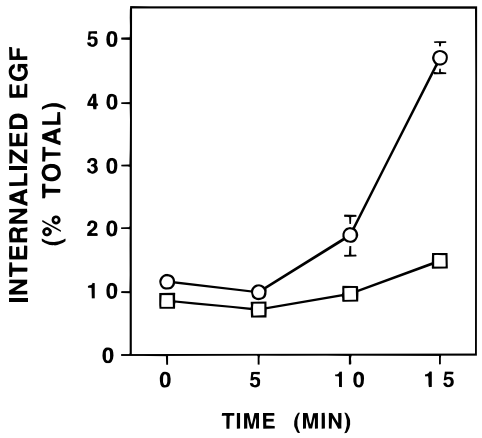

FIGURE 6: EGF internalization rate is reduced in cells expressing di-Ala658 EGFR. An equivalent number of serum-starved cells were incubated with ^{125}I-EGF for 2 h at 0 °C, warmed to 37 °C for the indicated times, and processed for quantitation of internalized ligand as described in Experimental Procedures. Wild type (○, 2WT) or di-Ala658 (□) mutated EGFRs were incubated with 0.4 nM ^{125}I-EGF. Error bars represent standard deviations from the mean of quadruplicate samples.

binding induced internalization in cells expressing either the di-Ala658 EGFR (Figure 6) or di-Ala679 EGFR (data not shown), but the rate of cellular EGF accumulation was reduced relative to that in cells expressing wild type EGFR. Internalization was retarded up to 60% for the di-Ala679 mutant, while it was retarded almost 90% in cells expressing the di-Ala658 mutant. The extent of inhibition of the internalization rate correlated with the ratio of truncated to full size receptors and their relative level of EGF-induced autophosphorylation.

Full-Length Di-Ala Receptors Are Degraded upon EGF Stimulation. To determine whether the loss of a di-leucine motif would affect the rate of EGF-induced degradation, the turnover of cell surface di-Ala658 receptors in response to

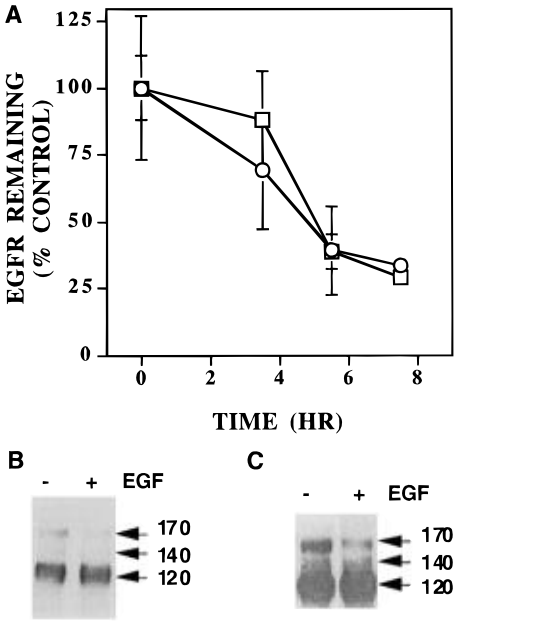

FIGURE 7: Effect of EGF treatment on di-Ala658 EGFR stability. Equivalent numbers of cells expressing wild type or di-Ala658 EGFRs were surface labeled by biotinylation and treated with 10 nM EGF for the indicated times, and the receptors were immunoprecipitated and analyzed as described in Experimental Procedures. (A) Plot showing loss of biotinylated 170 kDa EGFRs in response to EGF. The amount of biotinylated 170 kDa EGFR from cells expressing either wild type (○, 2WT) or di-Ala658 (□) EGFR was quantitated by densitometry. The data are from duplicate samples, and the deviation from the mean is shown by error bars. This result is representative of two independent experiments. (B) Autoradiogram showing surface biotinylated di-Ala658 EGFRs treated with or without 10 nM EGF for 5.5 h. (C) Longer exposure of autoradiogram shown in (B) to illustrate effects of EGF treatment on 140 and 170 kDa receptors. This result is representative of two independent experiments.

EGF was measured. Cells expressing either the wild type or the di-Ala658 mutant EGFRs were surface-labeled by biotinylation and then exposed to EGF for several hours. Both the wild type and mutant 170 kDa receptors were degraded almost completely by 7.5 h, with half-lives of approximately 5 h (Figure 7A). In contrast, the mutant truncated EGFRs were not significantly degraded even after 5.5 h of EGF treatment (Figure 7B,C). The full-length, surface-expressed di-Ala679 receptor, but not the truncated di-Ala679 species, was also significantly degraded in response to EGF (data not shown).

DISCUSSION

In order to understand the signals that are involved in regulating the EGFR, we investigated the role of two juxtamembrane di-leucines at residues 658 or 679 in regulating EGFR expression, activity, or degradation in stably transfected CHO cells. The results indicate that loss of either di-leucine motif leads to the generation of carboxy-truncated EGFRs that are expressed on the cell surface but do not undergo autophosphorylation or EGF-induced degradation. Thus, the juxtamembrane di-leucine motifs appear to be important for expression of full-length EGF receptors.

Both lysosomal and cell surface proteins are targeted for degradation via di-leucine motifs. Di-leucines are involved in the targeting of lysosomal enzymes such as the acid hydrolase cathepsin D through an association with the mannose-6 phosphate receptor (Johnson & Kornfeld, 1992).

In contrast to many other proteins, it appears that this sorting occurs intracellularly rather than from the plasma membrane. The internalization and down regulation of interleukin-6 (IL-6) and its receptor has been shown to be mediated through the IL-6 receptor associating signal transducer protein gp130 (Dittrich et al., 1994). This down regulation requires a di-leucine in the cytoplasmic domain of gp130. The insulin receptor also contains a number of cytoplasmic di-leucine motifs (Haft et al., 1994). Several of these di-leucines, when individually fused to the interleukin-2 receptor a chain (Tac antigen), as chimeric proteins, are sufficient to induce its lysosomal localization. These results indicate that di-leucine motifs can regulate the turnover of cell surface receptors.

The presence of di-leucines in the juxtamembrane region of the EGFR raises the possibility that these sites could play a role in receptor degradation. Receptors truncated at 958, which contain the juxtamembrane domain, can be targeted to lysosomes, consistent with a role for di-leucine motifs in this regulation (Herbst et al., 1994). However, the observation that the full-length di-Ala EGFRs undergo EGF-induced degradation suggests that neither of the di-leucine motifs is, by itself, required for targeting of receptors to lysosomes. It was not possible to determine whether loss of both di-leucine motifs would alter degradation, since no population of cells expressing the receptor with both these sites mutated could be isolated. Interestingly, mutation of Thr669, a residue situated between the di-Leu658 and di-Leu679, resulted in enhanced EGF-stimulated degradation (Morrison and Rosner, submitted). Thus, the residue at 669 is able to modulate lysosomal targeting, possibly through interaction with the surrounding di-leucine residues.

The conservative replacement of di-Ala for either di-Leu658 or di-Leu679 does not appear to significantly alter full-length EGF receptor structure. Similar substitutions at the neighboring Thr654 site block or reduce down regulation of the EGFR by PKC, indicating that this region is important in mediating the action of PKC (Countaway et al., 1990; Lund et al., 1990; Morrison et al., 1993). Yet, both the di-Ala658 and di-Ala679 170 kDa receptors resemble wild type EGFRs in their sensitivity to regulation by PKC, confirming that the di-Ala substitutions do not cause a major structural perturbation within the juxtamembrane region. In addition, the full-length di-Ala receptors are capable of undergoing autophosphorylation and degradation in response to EGF. Furthermore, the expression of the full-length di-Ala mutant receptors at the cell surface suggests that these sequences do not alter the normal transport of this protein. Thus, the full-length di-Ala mutant receptors do not appear to be significantly different from their wild type counterpart.

The differences in the extent of internalization between the mutant and wild type EGFRs are probably due, at least in part, to the presence of the terminally truncated 140 and 120 kDa receptors. A carboxy-truncated EGFR with only the first nine residues in the juxtamembrane region, when expressed in 3T3 cells, has been shown to bind ligand but not to undergo internalization (Felder et al., 1992). When truncated receptors of similar size to those described here were coexpressed with wild type EGFR, EGF treatment induced receptor heterodimerization (Kashles et al., 1991). In 3T3 cells transfected with both truncated and wild type receptors, EGF was internalized at a reduced rate even though the wild type EGFR was targeted to the lysosomes. In our system, the truncated di-Ala receptors are likely to be defective in internalization, since the 120 kDa receptor has lost the entire CAIN domain which contains several internalization signals, and the 140 kDa receptor truncation extends into and is likely to disrupt this internalization domain (Chen et al., 1989; Nesterov et al., 1995). If one assumed that only the 170 kDa receptor is internalized and corrected for the amount of EGF bound to this species, then the rate of EGF internalization of the 170 kDa mutant would be similar to that of the wild type receptor. These results suggest that the presence of the truncated di-Ala EGFRs can lower the overall internalization of ligand. It is also possible that loss of one of the di-leucine motifs in the EGFR can contribute to the reduction in EGF internalization but not degradation, as observed previously for the insulin receptor (Haft et al., 1994).

Heterodimerization could also lead to a reduction in EGF-induced kinase activity of the full-length di-Ala EGFRs. While the 140 kDa receptor may be an active kinase, it is unlikely that the 120 kDa receptor has kinase activity since its size indicates that it is truncated within the kinase domain. Dominant-negative suppression of kinase activity through heterodimer formation between kinase-inactive truncated receptors and full-length receptors has been described for the EGFR (Kashles et al., 1991) as well as other tyrosine kinases (Qian et al., 1994; Spivak-Kroizman et al., 1992). Since the reduction in level of autophosphorylation of the 170 kDa di-Ala EGFRs can be explained by the formation of kinase-inactive dimers with the 120 kDa receptors, it is possible that the full-length di-Ala homodimers have normal kinase activity.

The mechanism by which either of the di-Ala mutations leads to the generation and stable expression of similar carboxy-truncated receptors is not clear. The likely origin of these species is either from premature transcriptional termination or from immediate post-translational proteolysis. The observation that the wild type receptors also have truncated EGFRs of similar size raises the possibility that loss of either di-leucine enhances the generation and/or leads to stabilization of truncated receptors that are normal byproducts of EGFR expression in CHO cells. Whether the extent of EGFR truncation varies with cell type remains to be determined. Cellular mechanisms to remove improperly folded membrane proteins have been described previously. If membrane proteins are inappropriately folded, chaperone proteins as well as retention proteins (BiP) in the endoplasmic reticulum (Suzuki et al., 1991) provide either an environment to enable the correct folding to occur or the time to achieve a stable conformation. If proteins are aberrant and unable to form a stable structure, they are removed through degradation (Bonifacino et al., 1990a,b). This degradation occurs outside of the lysosome and is presumed to take place in the endoplasmic reticulum (Bonifacino et al., 1990b).

Consistent with this mechanism, loss of the di-leucines in the EGFR could impart an instability to the nascent protein chain, increasing the likelihood of proteolysis. The observation that each mutant produces different ratios of full-length to truncated receptors, suggests, however, that the extent of proteolysis must differ depending on which di-leucine motif is altered. The possibility that the truncated receptors are generated by limited proteolysis provides a potential explanation for our inability to generate EGFRs expressing the double di-alanine mutation. The double mutants may be too susceptible to proteolysis to be stably expressed. Independ-

ent of which mechanism is responsible, the generation from both mutants of truncated species with similar sizes and antibody recognition profiles suggests that a common mechanism exists. The observation that substitutions of the juxtamembrane di-leucine motifs lead to the generation of aberrant, carboxy-truncated receptors suggests that the fidelity of this region is critical for ensuring the expression of active, full-length EGF receptors.

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