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G1 cell cycle arrest due to the inhibition of erbB family receptor tyrosine kinases does not require the retinoblastoma protein

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Abstract

The erbB receptor family (EGFr, erbB-2, erbB-3, and erbB-4) consists of transmembrane glycoproteins that transduce extracellular signals to the nucleus when activated. erbB family members are widely expressed in epithelial, mesenchymal, and neuronal cells and contribute to the proliferation, differentiation, migration, and survival of these cell types. The present study evaluates the effects of erbB family signaling on cell cycle progression and the role that pRB plays in regulating this process. ErbB family RTK activity was inhibited by PD 158780 in the breast epithelial cell line MCF10A. PD 158780 (0.5 μM) inhibited EGF-stimulated and heregulin-stimulated autophosphorylation and caused a G1 cell cycle arrest within 24 h, which correlated with hypophosporylation of pRB. MCF10A cells lacking functional pRB retained the ability to arrest in G1 when treated with PD 158780. Both cell lines showed induction of $p27^{KIP1}$ protein when treated with PD 158780 and increased association of $p27^{KIP1}$ with cyclin E–CDK2. Furthermore, CDK2 kinase activity was dramatically inhibited with drug treatment. Changes in other pRB family members were noted with drug treatment, namely a decrease in p107 and an increase in p130. These findings show that the G1 arrest induced through inhibition of erbB family RTK activity does not require functional pRB.
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Keywords: Cell cycle arrest; erbB family; Retinoblastoma protein

Introduction

The erbB receptor family consists of four members: EGFr (erbB-1), erbB-2 (HER2 or neu), erbB-3, and erbB-4. These receptors are transmembrane glycoproteins that possess an extracellular ligand-binding domain, a single transmembrane region, an intracellular tyrosine kinase domain, and a cytoplasmic carboxy-terminal tail composed of specific tyrosine-containing sequences. Upon ligand binding, these receptors can dimerize or heterodimerize, which induces phosphorylation of the receptors at tyrosine residues in the carboxy-terminal tail. The phosphotyrosine sites in turn can recruit a diverse set of proteins in a sequence-specific fashion to initiate the transduction of extracellular signals to the nucleus [1–4].

The most extensively studied signaling pathway of the erbB receptor family is that involving EGFr-stimulated cell proliferation via Ras and MAP kinase activation. Activation of the EGF receptor by ligand binding leads to the recruitment of GRB2-Sos complexes to the phosphotyrosine sites in the carboxy-terminal tail of the receptor. Sos in turn activates Ras by causing it to exchange GDP for GTP. Ras then binds to the regulatory domain of Raf-1 and increases its kinase activity. Raf-1 phosphorylates and activates MEK, a kinase that phosphorylates and activates MAP kinase. Activated MAP kinase translocates to the nucleus where it phosphorylates various transcription factors and induces gene transcription and cell proliferation [1–3,5].

Signaling pathways activated by receptor tyrosine kinases, such as the Ras/Raf-1/MAP kinase pathway discussed above or others such as PI3-kinase, PLCγ, or the Jak-STATs, ultimately converge onto the cell cycle machinery to promote cell cycle progression and division [1,6]. One component of the cell cycle machinery that is transcriptionally activated by growth factor stimulation is

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the D-type cyclins [7,8]. These cyclins are thought to act as growth factor sensors since their induction is dependent on the presence of growth factors, and their decline occurs immediately after growth factor withdrawal [9,10]. When D-type cyclins are synthesized, they bind to a family of proteins called the cyclin-dependent kinases or CDKs. D-type cyclins can specifically associate with CDK6 or CDK4 to form holoenzymes in which the cyclin acts as the regulatory domain and the CDK acts as the catalytic domain. These holoenzymes when activated by specific phosphorylation/dephosporylation events are then capable of phosphorylating the appropriate substrates that promote progression of cells through the G1 phase of the cell cycle [11–13].

The primary substrate for the G1 cyclin–CDK protein complexes is the retinoblastoma tumor suppressor protein (pRB), which is the key regulator of the G1-to-S-phase transition. During the G1 phase of the cell cycle, pRB is hypophosphorylated and prevents cells from entering into S-phase. Hypophosphorylated pRB retains cells in G1 by binding and sequestering various transcription factors (e.g., E2F) that regulate transcription of genes needed for DNA synthesis. When G1 cyclin–CDK complexes (cyclin D–CDK6/4 and cyclin E–CDK2 protein complexes) phosphorylate pRB, pRB is inactivated, transcription factors are released, and cells are able to progress into S-phase [11,14,15].

Numerous groups have evaluated the effects of inhibiting EGF receptor signaling on cell cycle progression. Several studies have found that inhibiting EGFr tyrosine kinase activity or blocking EGFr ligand binding leads to a G1 arrest. The G1 arrest is associated with decreases in cyclin D1 and CDK4, induction of $p27^{KIP1}$ as well as CDK2-associated $p27^{KIP1}$, loss of CDK2 activity, and hypophosphorylated pRB [16–24]. Similar effects have been shown with inhibition of erbB2 receptor activity [25,26]. Although several of these papers showed the requirement of $P27^{KIP1}$ to induce a G1 cell cycle arrest when erbB receptor activity was inhibited [26,20], the role of pRB was not investigated. Because hypophosphorylated pRB could arise as an indirect consequence of a G1 cell cycle arrest, the present study investigates directly whether the cell cycle arrest caused by erbB receptor inhibition is dependent on pRB. Studies in cell lines containing functional pRB (MCF10A) and cells devoid of pRB (MCF10A CL6) demonstrated that inhibiting erbB family members caused cells to arrest in G1 within 24 h independent of pRB function. The G1 arrest correlated with induction of $p27^{KIP1}$ and formation of cyclin E–CDK2–$p27^{KIP1}$ protein complexes. Inhibition of both CDK4 and CDK2 kinase activity was detected after drug treatment. Interestingly, a decrease in p107 with a concomitant increase in hypophosphorylated p130 was also observed regardless of pRB status, suggesting that other pRB family members may play a role in regulating the G1 arrest induced by erbB receptor inhibition.

Materials and methods

Chemicals and reagents

PD 158780 (4-[ar(alk)ylamino] pyridopyrimidine) was synthesized as previously described [27]. EGF was obtained from the Chiron Corp. (Emeryville, CA), and a fragment of the heregulin-β_1 (Ser_{177}–Glu_{241}) was generated by recombinant methods as described [28]. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless specified.

Cell culture

The MCF10A breast epithelial cell line was purchased from the Karmanos Cancer Center, Wayne State University (Detroit, MI), and contained an empty vector possessing a neomycin resistance gene. MCF10A CL6 cells were clonally expanded from a single cell isolated from a population of MCF10A cells that spontaneously lost pRB protein expression after several passages in culture. MCF10A cells and MCF10A CL6 cells were maintained in DMEM/F-12 media (Gibco BRL, Bethesda, MD) containing 5% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT), 20 ng/ml EGF, 10 μg/ml insulin (Gibco BRL), 100 ng/ml cholera toxin, 500 ng/ml hydrocortisone, 10 μg/ml gentamycin (Gibco BRL), 1 mM $CaCl_2$, and 300 μg/ml Geneticin (Gibco BRL). Control cell lines (MDA-MB-468, MDA-MB-453, Jurkat and SKW6.4 cell lines) were purchased from American Type Culture Collection and grown in DMEM/F-12 medium supplemented with 10% fetal bovine serum and 10 μg/mL Geneticin. Cell cultures were maintained at 37°C in a humidified incubator containing 5% CO_2.

Inhibition of receptor autophosporylation

The ability of PD 158780 to inhibit EGF receptor family autophosphorylation in cultured cells was assessed by immunoblotting techniques. MCF10A cells were plated in six-well plates and grown to 80–90% confluence. Medium was removed from each well and replaced with DMEM/F-12 medium lacking serum and all other components described above. After cells were incubated for 22 h in this serum-free and growth factor-free media, they were treated with PD 158780 for 2 h to inhibit erbB tyrosine kinases. Cells were then stimulated for 5 min with either 20 ng/ml EGF or 10 ng/ml heregulin. Cells were immediately lysed in 0.25 ml of boiling Laemmli buffer (2% sodium dodecyl sulfate, 5% β-mercaptoethanol, 10% glycerol, and 50 mM Tris, pH 6.8) and scraped from wells. The collected cellular lysates were passed through a 25-gauge needle to shear DNA then heated at 100°C for 5 min. A 30 μl aliquot of each sample was loaded onto a pre-cast (4–20%) SDS-polyacrylamide gel (Novex, San Diego, CA). Proteins were separated by gel electrophoresis then transferred to a nitrocellulose membrane. Nitrocellulose blots were placed

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