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Tyrphostin ErbB2 Inhibitors AG825 and AG879 Have Non-specific Suppressive Effects on gp130/ STAT3 Signaling

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Although the interaction between gp130 and the ErbB family has frequently been shown in cancer cells, the mechanism of this interaction remains unclear and controversial. In the present study, we found that specific tyrphostin inhibitors of ErbB2 (AG825 and AG879), but not ErbB1 inhibitor (AG1478), suppressed IL-6-induced tyrosine phosphorylation of STAT3 in schwannoma cells. However, biochemical evidence for transactivation of ErbB2 by IL-6 was not observed. Additionally, the inhibition of ErbB2 expression, with either a specific RNAi or transfection of an ErbB2 mutant lacking the intracellular domain did not inhibit the IL-6-induced tyrosine phosphorylation of STAT3. Thus, it seems that tyrphostins, which are known as specific inhibitors of the ErbB2 kinase, may have non-specific suppressive effects on the IL-6/STAT3 pathway.

Key Words: ErbB2 kinase, Interleukin-6, gp130, Schwannoma, STAT3, Neuregulin

INTRODUCTION

Interleukin-6 (IL-6) is a pleiotrophic cytokine that has been implicated in various biological and pathological processes, such as inflammation, development and cardiovascular disease (Kamimura et al, 2003). IL-6 binds to the IL-6 receptor (IL-6R), and this IL-6-IL-6R complex then activates the signal transducing receptor gp130. Activated gp130 subsequently signals to signal transducer and activator of transcription 3 (STAT3), principally using the Janus tyrosine kinase, which can directly phosphorylate gp130 and STAT3 (Kamimura et al, 2003). The tyrosine-phosphorylated STAT3 acts as a transcription factor, subsequently inducing the expression of numerous genes involved in cell survival, apoptosis and differentiation (Battle and Frank, 2002).

Neuregulin-1 (NRG) stimulates proliferation in developing Schwann cells (Cheng et al, 1998), and ErbB2 and ErbB3, members of ErbB receptor tyrosine kinase family, are known receptors for NRG in Schwann cells (Carroll et al, 1997). These growth factor receptors have been implicated in the development of many human cancers, including Schwann cell tumors (Hynes and Lane, 2005; Stonecypher et al, 2005). For example, aberrant activation of the ErbB2 kinase is associated with the development of malignant peripheral nerve sheath tumors (Stonecypher et al, 2005), and transgenic mice highly expressing NRG develop Schwann cell neoplasms (Huijbregts et al, 2003). Thus, the inhibition of ErbB2 kinase activity seems to an important approach for the treatment of peripheral nerve sheath tumors and other ErbB2-overexpressing cancers (Hynes and Lane, 2005).

There are several previous studies to demonstrate cross-talk between gp130 and ErbB2, especially in cancer cells. For example, IL-6 has been shown to transactivate ErbB2 in prostate cancer cells (Qiu et al, 1988), and ErbB2 has been shown to associate with gp130 both constitutively and in an IL-6-dependent fashion (Qiu et al, 1988; Grant et al, 2002). However, the physiological relevance and molecular mechanism of this cross-talk remain unclear. In the present study, we investigated possible interactions between ErbB2 and IL-6 signaling in Schwann cells and found that tyrphostin-type ErbB2 kinase inhibitors blocked the IL-6/STAT3 signaling pathway. Interestingly, however, we found no biochemical evidence to support the interaction between ErbB2 and gp130.

METHODS

Materials

Restriction enzymes and T4-DNA ligase were purchased from New England Biolabs (NEB, Ipswich, MA). Media and sera for cell culture were purchased from Life Technologies (Grand Island, NY). All phospho-specific antibodies and recombinant IL-6 were purchased from Cell Signaling Technology (Beverly, MA). Anti-gp130, STAT3, EGFR and ErbB2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Reagents for electrophoresis and Western blot analysis were purchased from BioRad (Richmond, VA). Goat antibodies to gp130 and IL-6R were obtained from

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ABBREVIATIONS: IL-6, interleukin-6; STAT3, signal transducer and activator of transcription 3; NRG-1, neuregulin-1; IL-6R, interleukin-6 receptor; EGFR, epidermal growth factor receptor.

R&D systems (Minneapolis, MN). AG1478, AG825, AG879 and GW583340 were obtained from Calbiochem (Gibbstown, NJ) and Sigma (Saint Louis, MO).

Cell culture and drug treatment

The schwannoma cell line RT4, MCF-7 breast cancer cells, and U87 glioma cells were obtained from the American Type Culture Collection (Rockville, MD) and were maintained as previously described (Lee et al, 2007) or according to ATCC protocols. Cells were starved for 4 h or overnight in serum-free DMEM supplemented with 0.1% fetal bovine serum (FBS) before addition of cytokines. For the treatment with ErbB2 inhibitors, drugs were applied 30 min before addition of IL-6 and maintained until the end of the experiment.

DNA binding activity assay

Total cellular extracts were prepared from RT4 cells using lysis buffer conatining 15 mM HEPES, pH 7.6, 40 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 5% glycerol. Extracts (300 μg) were incubated with 1.5 μg of a STAT3-interacting gel shift oligonucleotide (5'-GATCCTTCTGGG AATTCCTAGATC-3') conjugated to agarose beads (Santa Cruz) for 2 h at room temperature (Ng et al, 2006). After incubation, the beads were extensively washed with lysis buffer. Precipitated proteins were eluted from the beads with SDS sample buffer, resolved by SDS-PAGE, and then transferred to a nitrocellulose membrane for immunoblotting.

Generation of an ErbB2 truncation mutant

An ErbB2 truncation mutant lacking the intracellular domain (ErbB2TR; 1aa~675aa) was generated via PCR using full length human HER2 cDNA in the pCEP4 vector. The following primers were used for amplification: 5'- aagcttacatggagctggcggccttg-3' (forward), 5'-ctcgagcttctgctgccgtcgctt-3' (reverse). As seen from these sequences, the forward primer contained a Hind III site, and the reverse primer contained an Xho I site. After PCR amplification and restriction enzyme digestion, the PCR fragments were inserted into the AP5 vector (Lee et al, 2006), which contains Myc and His tags. The sequence of the construct was verified by sequencing analysis in an ABI 3700 automated sequencer (Macrogen, Seoul).

Preparation of adult Schwann cells

Schwann cells from adult sciatic nerves were cultured as previously described (Lee et al, 2007). Briefly, the axotomized sciatic nerves were removed under aseptic conditions. Nerves were washed twice in calcium-and magnesium-free Hanks' balanced salt solution (CMF-HBSS), and teased apart. Then, the nerves were incubated in 0.2% collagenase in CMF-HBSS at 37°C for 2 h, and nerves were dissociated by gentle shaking followed by two or three triturations. Cells were centrifuged, and then the pellet was dissociated in prewarmed DMEM containing 10% fetal bovine serum (FBS). They were grown on poly-L-lysine coated dishes (100 μg/ml).

Immunoprecipitation and Western blot analysis

Cell lysates were prepared with modified RIPA buffer and centrifuged at 10,000 g for 10 min at 4°C. For immunoprecipitation, 1 mg of cellular proteins was incubated overnight with primary antibodies (1 μg/ml) and protein A/G-conjugated agarose beads at 4°C. The immune-complexes were centrifuged at 1,500 rpm for 2 min at 4°C, washed 3 times with modified RIPA buffer, and then subjected to 8% SDS-PAGE. Western blot analysis was performed using standard techniques previously described (Lee et al, 2006; 2007). For quantification, X-ray films were then scanned using a HP scanner and analyzed with LAS image analysis system (Fujifilm, Japan). The intensity of pSTAT3 bands was normalized to that of STAT3 from three independent experiments.

Transfection and Immunofluorescent labeling

Cells were transfected with expression constructs using Lipofectamine 2,000 transfection reagent (Invitrogen) according to the manufacturer's instructions. Two days after transfection, cells were treated with IL-6 for 20 min, and pSTAT3/ Myc double immunofluorescent staining was then performed. RT4 cells were fixed with ice-cold methanol for 15 min at 4°C and then washed three times with phosphate buffered saline (PBS). Fixed cells were incubated overnight with an anti-phospho-STAT3 antibody (1 : 200) and a monoclonal anti-Myc antibody (1 : 1,000) in PBS containing 0.1% Triton X-100 at 4°C. After washing with PBS, cells were incubated with Alexa 488-conjugated anti-rabbit IgG and Cy3-conjugated anti-mouse IgG (1 : 1,000; Molecular probes) for 2 h at room temperature, and were viewed under a laser confocal microscope (LSM510, Carl Zeiss, Germany). For quantitative assays, the number of pSTAT-positive nuclei from randomly selected Myc-positive cells was counted. All experiments were repeated at least three times, and 200~250 cells were counted from each experiment.

RNA Interference

ErbB2 RNA interference (RNAi) oligonucleotides were provided by Dharmacon Research (Chicago, IL). Transient transfection of ErbB2 RNAi was performed with the Dharma FECT transfection reagent (Dharmacon Research), according to the accompanying protocol. After transfection, cells were allowed to recover for 2 days and treated with IL-6 for 20 min.

Statistical analysis

Differences in the means between the treatment groups were statistically assessed using an analysis of variance, followed by the Bonferroni post hoc test. Differences were considered to be statistically significant if $p<0.05$.

RESULTS

Effects of specific ErbB2 inhibitors on IL-6-induced STAT3 activation

RT4 schwannoma cells were stimulated with IL-6 (50 ng/ml) for 20 min in the absence or presence of an ErbB1 inhibitor (AG1478) or ErbB2 inhibitors (AG825, AG879). As shown in Fig. 1A, B and Fig. 2B, treatment of the cells with these tyrphostin ErbB2 inhibitors inhibited IL-6-in-

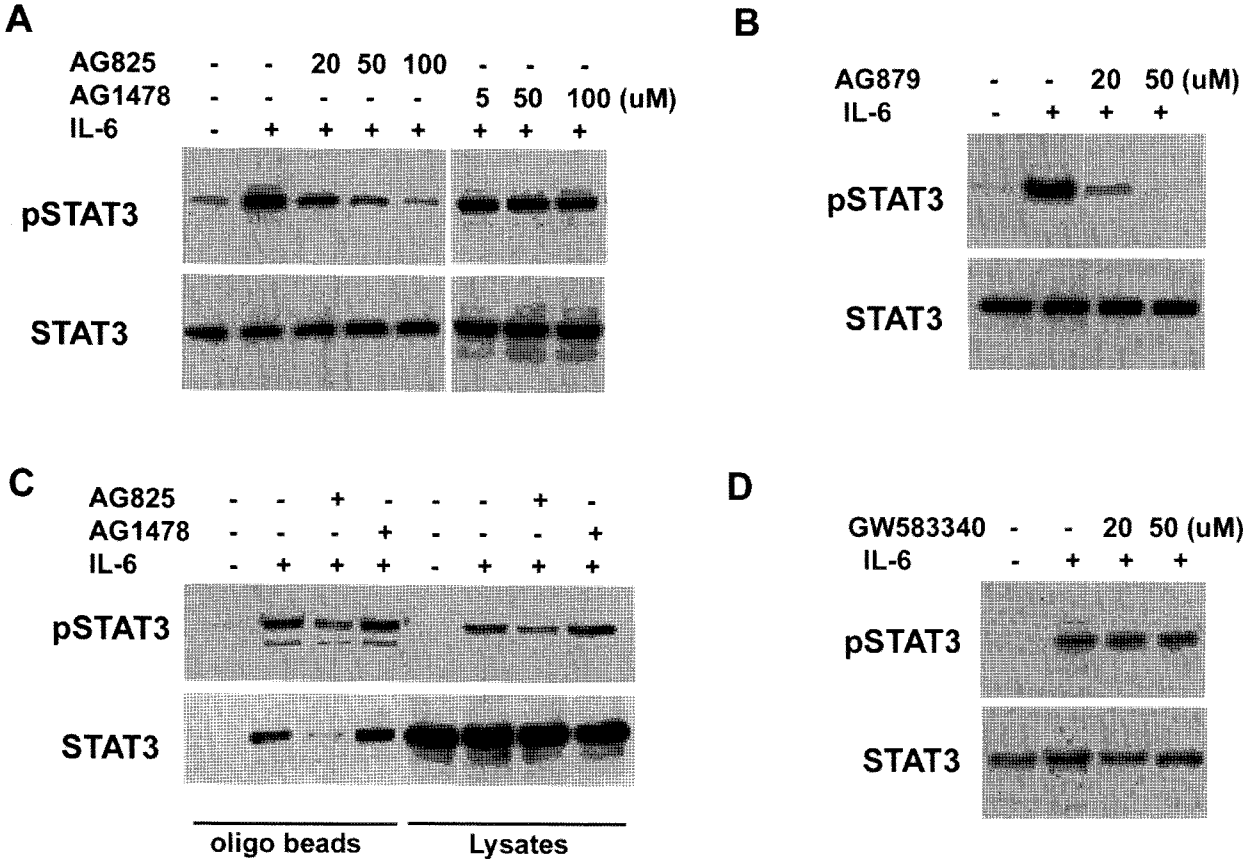

Fig. 1. Specific tyrphostin ErbB2 inhibitors block IL-6-induced tyrosine phosphorylation of STAT3. (A, B) The effects of an ErbB1 inhibitor (AG1478) or ErbB2 inhibitors (AG825, AG879) on IL-6- induced tyrosine phosphorylation of STAT3 in RT4 cells were analyzed. ErbB2 inhibitors dose-dependently reduced tyrosine phosphorylation of STAT3 induced by IL-6. (C) STAT3 DNA binding activity was analyzed using a STAT3-interacting oligonucleotide conjugated to agarose beads, after 20 min of IL-6 treatment. STAT3 was immunoprecipitated and analyzed via immunoblotting, with antibodies against pSTAT3 or STAT3. (D) An ErbB1/ErbB2 dual inhibitor, GW583340, did not block IL-6-induced tyrosine phosphorylation of STAT3.

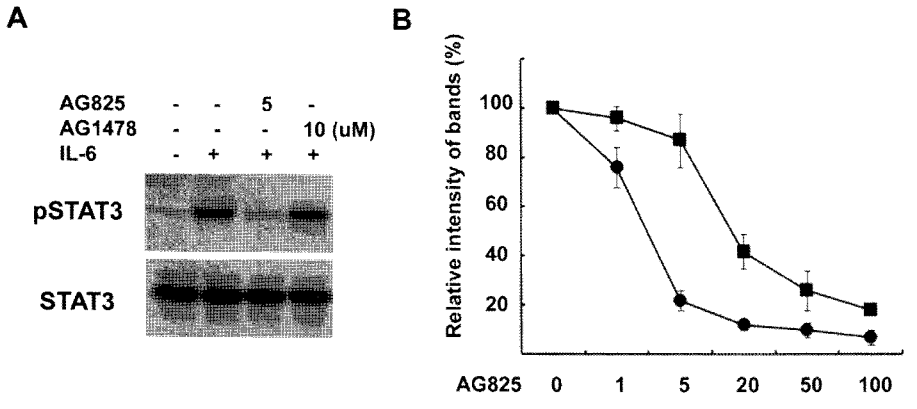

Fig. 2. Effects of ErbB1 and ErbB2 inhibitors on IL-6-induced tyrosine phosphorylation of STAT3 in primary Schwann cells. (A) The selective inhibition of IL-6-induced (20 min) tyrosine phosphorylation of STAT3 by AG825 was demonstrated in primary Schwann cells. (B) Dose-dependent curves showing the effect of AG825 on RT4 (■) cells and primary Schwann cells (●). The intensity of IL-6- stimulated pSTAT3 band in the absence of AG825 was set as 100% of relative intensity. The curve shows that primary Schwann cells were more sensitive to AG825 than RT4 cells.

duced tyrosine phosphorylation of STAT3 in a dose-dependent manner, without affecting STAT3 protein levels (IC50; ~15 μM). In contrast, a specific inhibitor of ErbB1 kinase, AG1478, did not block tyrosine phosphorylation of STAT3 induced by IL-6 (Fig. 1A). We also tested whether AG825 inhibited the IL-6-induced DNA binding activity of STAT3, and the result showed that AG825 significantly suppressed the DNA binding activity of STAT3 induced by IL-6 (Fig.

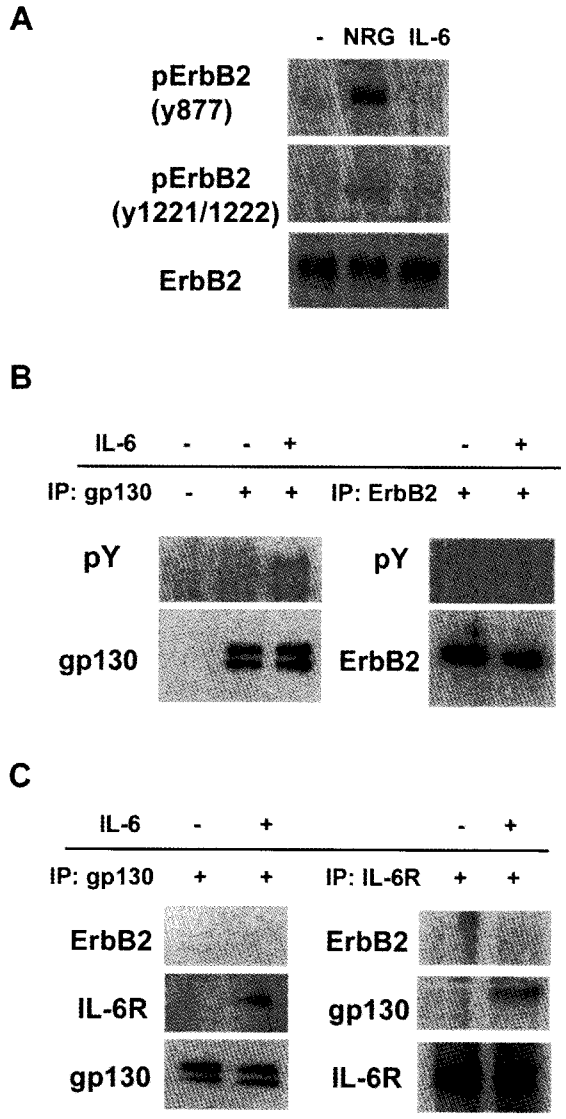

Fig. 3. IL-6 does not transactivate ErbB2 in Schwann cells. (A) Western blot analysis with anti-phospho-ErbB2 antibodies showed that IL-6 did not induce tyrosine phosphorylation of ErbB2 at tyrosines 877, 1221 or 1222, whereas NRG induced strong tyrosine phosphorylation of ErbB2 at tyrosine 877 and mild phosphorylation at tyrosines 1221 and 1222. (B) Cell lysates were subjected to immunoprecipitations using either an anti-gp130 antibody or an anti-ErbB2 antibody, and then immunoblotting was performed using an anti-phosphotyrosine antibody (4G10, pY). IL-6 did not induce tyrosine phosphorylation of ErbB2, while it did stimulate the tyrosine phosphorylation of gp130. (C) Cell lysates were subjected to immunoprecipitations using either an anti-gp130 antibody or an anti-IL-6R antibody, and then immunoblotting was performed using an antibody to ErbB2 to demonstrate interactions between gp130 and ErbB2 or between IL-6R and ErbB2. IL-6 induced an interaction between IL-6R and gp130, but did not induce an interaction between ErbB2 and gp130 or IL-6R.

1C). On the other hand, the ErbB1 kinase inhibitor had no effect. These findings indicated that ErbB2 kinase activity may be required for IL-6-induced STAT3 activation.

We next tested the effect of a structurally unrelated dual

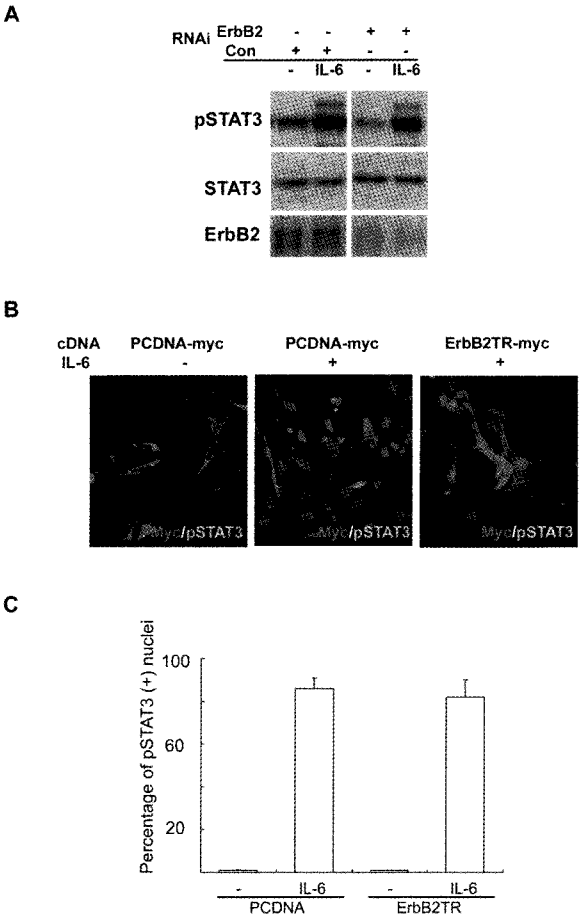

Fig. 4. Specific ablation of ErbB2 function does not inhibit IL-6-induced tyrosine phosphorylation of STAT3. (A) Cells were transfected with control siRNA or ErbB2 siRNA, and IL-6-induced tyrosine phosphorylation of STAT3 was analyzed using Western blot analysis. The suppression of ErbB2 expression with a specific RNAi did not affect the IL-6-induced tyrosine phosphorylation of STAT3. (B) Cells were transfected with an ErbB2 mutant lacking the intracellular domain (ErbB2TR) or empty vector. Two days after transfection, cells were incubated with IL-6 for 20 min, fixed and double-immunostained with an anti-Myc antibody and an anti- pSTAT3 antibody. (C) Quantitative analysis was performed by calculating the percentage of pSTAT3-positive nuclei among Myc- positive cells. Means±S.E from three independent experiments are shown.

ErbB1/ErbB2 inhibitor, GW583340. Unexpectedly, this potent dual inhibitor did not inhibit IL-6-induced STAT3 phosphorylation (Fig. 1D), however, it blocked EGF-induced ERK activation in U87MG glioma cells (data not shown). This finding led us to further question the role of ErbB2 in IL-6-induced STAT3 activation.

Effects of ErbB2 inhibitors on IL-6 signaling in primary Schwann cells

Since ErbB2-mediated neuregulin signaling and IL-6 signaling are important for Schwann cell physiology in normal and pathological peripheral nerves (Chen et al, 2007), we extended our studies to primary Schwann cells cultured from adult sciatic nerves. Thus, we tested the effects of

AG825 and AG1478 on IL-6-induced STAT3 activation in primary Schwann cells, and found that primary Schwann cells responded to AG825 at a lower concentration than that used in RT4 cells (IC50; ~2.5 μM, Fig. 2A, B). Nevertheless, the ErbB1 inhibitor AG1478 had no effect.

Biochemical evidence does not support transactivation of ErbB2 by IL-6

It was previously reported that IL-6 transactivates ErbB2 in prostate cancer cells (Qiu et al, 1998), and this finding led us to investigate whether IL-6 induced transactivation of ErbB2 activates the STAT3 pathway in Schwann cells. Thus, we investigated the transactivation of ErbB2 by IL-6 in primary Schwann cells using phospho-specific antibodies recognizing active ErbB2. Surprisingly, we found that IL-6 treatment for 10 min did not induce phosphorylation of tyrosines 877, 1221 or 1222 of ErbB2. However, NRG did induce phosphorylation of these tyrosine residues within 5 min, as expected (Fig. 3A). We also analyzed tyrosine phosphorylation of immunoprecipitated ErbB2 following IL-6 treatment of schwannoma cells, and found no induction of ErbB2 tyrosine phosphorylation by IL-6 (Fig. 3B). In contrast, however, IL-6 induced tyrosine phosphorylation of its own receptor, gp130. These findings indicate that IL-6 does not transactivate ErbB2 in Schwann cells.

We next examined possible IL-6-induced interaction between gp130 and ErbB2. However, we were unable to demonstrate an interaction between gp130 and ErbB2 after IL-6 treatment in RT4 cells (Fig. 3C). On the other hand, IL-6 induced formation of an IL-6R-gp130 complex within 5 min of IL-6 treatment. We further examined whether interaction between IL-6R and ErbB2 might occur following IL-6 treatment (Fig. 3C). By co-immunoprecipitation, we found that IL-6R did not form a complex with ErbB2, but did complex with gp130 following IL-6 treatment for 5 min. Thus, it seems unlikely that IL-6 transactivates ErbB2 in Schwann cells.

Transfection of ErbB2 RNAi or an ErbB2 mutant lacking the intracellular domain does not impair IL-6-induced tyrosine phosphorylation of STAT3

The absence of ErbB2 transactivation by IL-6 seems incompatible with the pharmacological results of AG825 and AG879 treatment, but seems to be in line with the negative effect of GW583340. To clarify this, we attempted to demonstrate a role of ErbB2 in the IL-6-induced tyrosine phosphorylation of STAT3, using two independent methods. We first employed siRNA to suppress the expression of ErbB2 in RT4 cells. Thus, cells were transfected with an ErbB2-specific RNAi or scrambled RNAi and were then stimulated with IL-6 for 20 min. As shown in Fig. 4A, we failed to observe any noticeable reduction in IL-6-induced STAT3 tyrosine phosphorylation following RNAi knockdown of ErbB2, even though ErbB2 expression was strongly suppressed by the RNAi. This finding indicated that ErbB2 may not be required for IL-6-induced tyrosine phosphorylation of STAT3 in schwannoma cells.

We next attempted to confirm this conclusion using a dominant negative form of ErbB2. We generated a truncated ErbB2 cDNA lacking the sequence coding for the intracellular domain of ErbB2 and analyzed the effects of expression of this truncated ErbB2 mutant on IL-6-induced nuclear translocation of pSTAT3, using immunofluorescent staining. Thus, transiently transfected cells were stimulated with IL-6 for 20 min, and then cells were double-immunostained with an antibody against pSTAT3 and an antibody against Myc-tag. We found that expression of the truncated ErbB2 protein did not affect IL-6-induced tyrosine phosphorylation of STAT3 (Fig. 4B, C).

DISCUSSION

While the idea of cross-talk between gp130 and ErbB2 has been addressed in the context of IL-6-induced ErbB2 transactivation in prostate cancer cells (Qui et al, 1988), the molecular mechanism and physiological relevance of this interaction remain uncertain. In contrast to the early finding of Qui et al. (1988), Badache and Hynes (2001) reported no evidence of ErbB2 transactivation by IL-6 in breast carcinoma cells such as T47D and MCF7, and IL-6-induced downstream signaling was found to be not altered by the suppression of ErbB2 activity. Furthermore, they showed the same negative result with prostate cancer cells. Even though Grant et al. (2002) found a constitutive interaction between gp130 and ErbB2, transactivation of ErbB2 by gp130 cytokines was not observed. Thus, further studies on the cross-talk between two receptors are clearly required. In the present study, we tried to demonstrate cross-talk between gp130 and ErbB family in terms of the STAT3 activation pathway in Schwann cells. Our result using two different pharmacological reagents showed contradictory results; although gp130/STAT3 signaling was inhibited by the tyrphostin ErbB2 inhibitors (AG compounds), another ErbB2 inhibitor GW583340 could not inhibit the signaling. Moreover, we failed to observe any evidence for IL-6-induced transactivation of ErbB2, and constitutive or IL-6-induced interaction between ErbB2 and gp130 was not found in Schwann cells. Finally, the activation of STAT3 by IL-6 was not altered at all by removing ErbB2 kinase activity with specific RNAi or dominant negative ErbB2 mutant. Therefore, it seems that the molecular mechanisms underlying cross-talk between gp130 and ErbB2 are more complicated than originally suggested or the effects of tyrphostin ErbB2 inhibitors on STAT3 signaling may be non-specific. Further studies are required in order to fully understand the complexity of this interaction.

Amplification of the ErbB2 gene has been found in many breast and gastrointestinal cancers, where it contributes to malignant tumor formation (Hynes and Lane, 2005). Selective tyrosine kinase inhibitors, that preferentially inhibit ErbB2 and are known as tyrphostins, have been developed and used for anticancer treatment and scientific research (Gazit et al, 1991; Osherov et al, 1993; Badache and Hynes et al, 2001; Hynes and Lane, 2005). Despite the well-known specificity of the tyrphostins, non-specific actions of these compounds have also been reported. For example, the ability of one EGFR inhibitor to up-regulate the retinoic acid receptor was shown to be independent of its ability to inhibit EGFR (Grunt et al, 2007), and AG1478 is known to inhibit potassium channel activity in addition to inhibiting ErbB1 (Choi et al, 2002). It should also be noted that AG879 is commonly used as a TrkA inhibitor (Xie et al, 2000). Thus, it seems plausible that AG825 and AG879 might possibly inhibit a crucial protein kinase or phosphatase involved in the IL-6 signaling pathway. Inhibitors of JAK2 or Src family kinases did not block IL-6-induced tyrosine phosphorylation in Schwann cells, as the ErbB2 inhibitors did (data

not shown), suggesting that the effects of the ErbB2 inhibitors are not mediated by non-specific inhibitory effects on JAK2 or Src family kinases. However, until the interplay between these pathways can more fully be elucidated, pharmacological results based on the use of ErbB2 inhibitors should cautiously be interpreted.

STAT3 has been shown to often be activated in several cancers such as glioma, breast and skin cancers (Rahaman et al, 2002; Chan et al, 2004; Selander et al, 2004). Furthermore the inhibition of constitutively active STAT3 has beeb reported to induce apoptosis and prevents malignancy formation (Rahaman et al, 2002; Selander et al, 2004; Iwamaru et al, 2007). Thus, the inhibition of STAT3 may be a new tumor-selective strategy for treatment of cancers. In the present study, we observed that tyrphostin ErbB2 inhibitors, AG825 and AG879, suppressed IL-6-induced STAT3 activation in schwannoma cell line, suggesting that the cytotoxic effect of tyrphostin inhibitors (Tsai et al, 1996) on tumor cells may in part be attributed to their non-specific suppressive effects on STAT3. In order to extend our present findings of a possible anti-cancer effect of the tyrphostin inhibitors, it seems necessary to investigate the effects of AG825 and AG879 on STAT3 activation in several STAT3-activated cancer cells. It may be possible that the tyrphostin inhibitors have an advantage of targeting both ErbB2 and STAT3 with potentially enhancing the anticancer effects.

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