PEA3 cooperates with c-Jun in regulation of *HER2/neu* transcription

KOSHI MATSUI[1], KAZUHITO SUGIMORI[2], HIRAKU MOTOMURA[2], NAOKO EJIRI[3], KAZUHIRO TSUKADA[1] and ISAO KITAJIMA[3,4]

[1]Second Department of Surgery, Departments of [2]Orthopedic Surgery, [3]Clinical and Molecular Pathology, [4]21st Century COE Program, Faculty of Medicine, University of Toyama, Toyama 930-0194, Japan

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Abstract. *HER2/neu* overexpressing breast tumors exhibit an increase in polyomavirus enhancer activator 3 (PEA3) expression. We examined the relationship between *HER2/neu* transcriptional activation and PEA3 in cooperation with c-Jun. *HER2/neu* promoter activity was decreased by deleting PEA3 binding site, and was downregulated when the PEA3 binding site was mutated. PEA3 and c-Jun each weakly enhanced luciferase expression of the *HER2/neu* promoter. However, the *HER2/neu* promoter response to PEA3 was considerably enhanced by c-Jun. Thus, we examined the interaction of PEA3 with c-Jun by the two-hybrid system, the transcriptional activity of PEA3 was specifically enhanced by c-Jun. When PEA3, c-Jun and coactivator p300 were cotransfected in MCF7 cells, the transcriptional activity of *HER2/neu* was increased by up to 20-fold. PEA3 and c-Jun-induced transcription of *HER2/neu* promoter was repressed by cotransfection of the dominant negative of p300. These results suggest that PEA3 and c-Jun stimulated synergistically the *HER2/neu* gene transcription with p300.

Introduction

The *HER2/neu* (also known as *c-erbB2*) gene encodes a 185-kDa transmembrane receptor with tyrosine kinase activity, which belongs to a family of receptors for the human epidermal growth factor. The amplification and the overexpression of *HER2/neu* are found in 20-30% of human breast cancers, and the increased expression is associated with poor prognosis (1). The *HER2/neu* signal is related to cell proliferation and transformation. As a result of *HER2/neu* receptor activation, downstream signals are channeled through adaptor proteins and exchange factors to mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and stress pathways involving the activation of c-Jun N-terminal kinase (JNK) (2). Downstream of the *HER2/neu*-Ras-Raf signaling cascade is MAPK, which induces the phosphorylation and resultant activation of many transcription factors.

Polyomavirus enhancer activator 3 (PEA3) is the founding member of the PEA3 subfamily of the *Ets* gene, which also includes ER81 and ERM. These genes share >95% sequence identity in the DNA binding domain and >60% sequence identity in the amino- and carboxy-terminal regions, which are known to be transcriptional activating domains (3). The *Ets* proteins share conserved winged helix-turn-helix *Ets* DNA-binding domains comprising ~85 amino acids. DNA binding is achieved by the interaction between the ETS domain and an ~10-bp sequence element termed the *Ets* binding site that comprises a highly conserved central core sequence, 5'-GGA (A/T)-3' (4). The PEA3 binding sites have been shown to be required for the expression of many genes important for migration and metastasis, such as cyclooxygenase-2 (COX-2) (5-7); gelatinases (8); stromelysin (9); matrilysin (MMP-7) (10,11); interleukin-8 (IL-8) (12); osteopontin (13); tissue inhibitor of metalloproteinase-1 (TIMP-1) (14); urokinase-type plasminogen activator (uPA) (15); and *HER2/neu* (16-19). Ninety-three percent of the *HER2/neu* overexpressing breast tumors, which represent 20-30% of all breast tumors, over-express PEA3 as well (20,21). Analysis of the *HER2/neu* promoter has revealed various DNA binding sites for transcription factors (19). PEA3 stimulates *HER2/neu* gene transcription by binding to sites at the *Ets* binding site of the *HER2/neu* promoter (19,20). Several studies have indicated that PEA3 plays a critical role in *HER2/neu*-mediated tumorigenesis (22-24), and that *HER2/neu* increases the activation of *Ets*, activator protein 1 (AP-1) and nuclear factor κB (NF-κB) (25). PEA3 is also activated by *HER2/neu*, Ras and Raf through MAPK pathways (26). Therefore, the *HER2/neu*-mediated activation of Ras and Raf signaling pathway components suggests that this pathway may play an important role in breast cancer.

Transcription factors cooperate with other transcription factors to achieve efficient gene activation. For example, Ets-1 and Ets-2 can bind to the PEA3 site and cooperate with exogenously expressed c-Fos and c-Jun (27). The involvement of closely spaced PEA3/AP-1 elements has been reported in several inducible genes including COX-2 (6), MMP-7 (11), uPA (15), and gelatinases (8). We have previously reported

Correspondence to: Dr Isao Kitajima, Department of Clinical and Molecular Pathology, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan
E-mail: kitajima@med.u-toyama.ac.jp

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that both PEA3 and AP-1 are required for constitutive IL-8 gene expression (12). Thus, the *Ets* family members often require cooperation with AP-1 for the selective and efficient activation of their target genes. The synergistic collaboration between PEA3 and AP-1 proteins has been shown to regulate the promoters of multiple genes associated with tumor progression. This apparent need for cooperation in regulating the expression of many tumor-associated genes suggests that this cooperation is also important for both PEA3 and AP-1 proteins to activate *HER2/neu* transcription.

Given the diverse and critical gene programs controlled by development-specific and tissue-restricted transcription factors, in particular, the members of the *Ets* family, efforts to fully understand the structural and functional regulators of *HER2/neu* promoter activity are necessary not only to better define the earliest *HER2/neu* dysregulating mechanisms associated with oncogene activation and malignant transformation, but also to develop novel and specific *HER2/neu* oncogene-targeted cancer therapeutics.

We show that the c-Jun activates PEA3, moreover, PEA3, c-Jun and p300 activate synergistically the *HER2/neu* gene transcription. Our results suggest that PEA3 and c-Jun stimulated synergistically the *HER2/neu* gene transcription with p300.

Materials and methods

Cell culture. MCF7 (Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University) cells and MDA-MB453 (American Type Culture Collection) cells were maintained in RPMI-1640 (Sigma-Aldrich Corp.) with 10% fetal bovine serum. These cells were grown at 37˚C in a humidified 5% CO_2 atmosphere.

Plasmids. The PEA3 expression vector (pcDNA3-PEA3) was a gift of Mien-Chie Hung (M.D. Anderson Cancer Center, TX). The c-Jun and dominant-negative expression vectors (c-Jun and TAM67) were gifts of Michael J. Birrer (National Institutes of Health). The amino-terminal deletion mutants were derived from pcDNA3-PEA3 by polymerase chain reaction amplification using suitable primers, followed by molecular cloning of the product into EcoRI and XhoI of FLAG-tagged pcDNA3 (Invitrogen Corp.). The primers used for deriving the amino-terminal deletion mutants all comprised an EcoRI site. The *HER2/neu* reporter plasmid (pNulit) was a gift of Mien-Chie Hung. The p300 and dominant-negative expression vectors (pCMV N HA p300 and p300 dCH3 33) were gifts of Marc Montminy (The Salk Institute, San Diego, CA).

Transfection and luciferase assay. Cells were transfected with 1 μg of expression vector, 1 μg of firefly luciferase reporter, and 100 ng of the Renilla luciferase internal control, SV40-RL (Promega Corp.), using Lipofectamine 2000 (Invitrogen Corp.) according to the manufacturer's protocol. The total amount of DNA was maintained at 4 μg by adding an empty vector (FLAG/pcDNA3). Luciferase activity was measured using a dual luciferase assay system (Promega Corp.).

Western blotting. Cells were collected from a 6-well plate, washed with PBS, and lysed on ice for 30 min with 500 μl of lysis buffer per well (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40) containing 1% aprotinin (Bayer) and 1 mM phenylmethylsulfonyl fluoride, and clarified by centrifugation. After normalization of protein content according to the

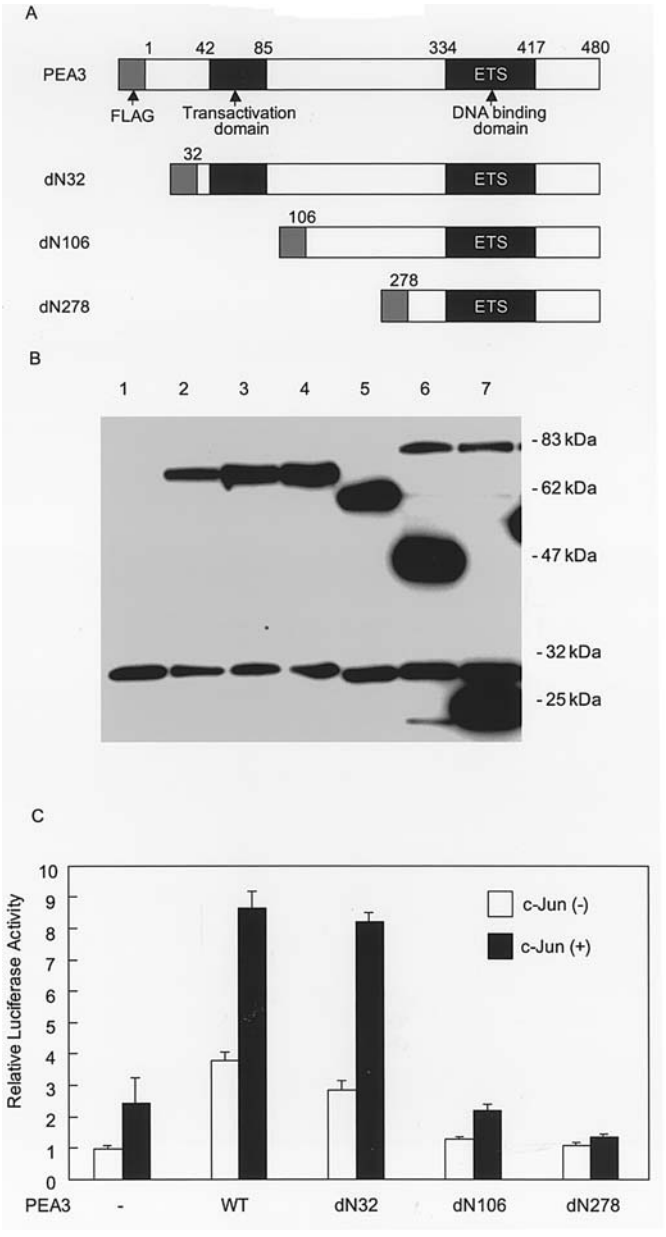

Figure 1. (A) Schematic representation of a series of truncated PEA3 constructs. The N-terminal transactivation domain is indicated by a grey box and the ETS DNA binding domain, by a black box. (B) Representative immunoblot illustrating the abundance of PEA3 and N-terminal deletion mutants in equal amounts (30 μg) of total cell protein from transfected MCF7 cells. Lane 1, lysate from MCF7 cells transfected with 1.0 μg of the empty vector, FLAG/pcDNA3. Lanes 2-4, lysate from MCF7 cells transfected with 0.25, 0.5, or 1.0 μg of FLAG/pcDNA3-PEA3 encoding WT PEA3. Lanes 5-7, lysate from MCF7 cells transfected with 1.0 μg of FLAG/pcDNA3-PEA3 encoding the N-terminal deletion mutants, dN32, dN106, and dN278, respectively. The monoclonal antibody, M2, was used for detecting FLAG-tag. (C) The *HER2/neu* promoter plasmid was cotransfected with combinations of the indicated PEA3 constructs and c-Jun expression vector into MCF7 cells. Data are presented as fold induction relative to cotransfection of the reporter with an empty vector. These were normalized to the RL-SV40 internal control. Data shown are means and SD of duplicate samples from one representative experiment. The experiment was performed three times, and similar results were obtained in each case.

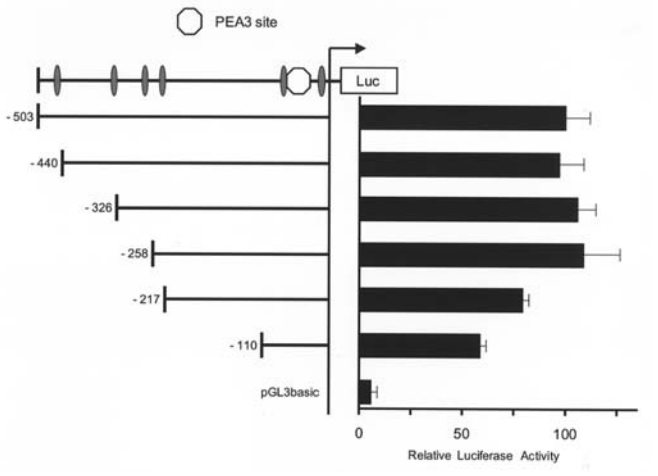

Figure 2. The indicated *HER2/neu* promoter fragments were cloned into pGL3-basic. The reporter gene constructs were transiently transfected into MDA-MB453 cells. The transcriptional activity of each construct was calculated relative to the luciferase activity observed with the full length of the *HER2/neu* promoter (-503/+30), which was set at 100, and normalized to RL-SV40 internal control. Data shown are means and SD for duplicate samples from one representative experiment. The experiment was performed three times, and similar results were obtained in each case.

DC protein assay (Bio-Rad), 30 μg of each protein sample was boiled for 3 min in SDS sample buffer (100 mM Tris-HCl, pH 8.8, 0.01% bromophenol blue, 36% glycerol, 4% SDS) containing 10 mM dithiothreitol. The samples were subjected to SDS-8.5% polyacrylamide gel electrophoresis. Proteins were transferred onto PVDF membrane (Bio-Rad). The membrane was blocked for 1.5 h with 5% nonfat dry milk in TBS containing 0.1% Tween-20 and incubated overnight with the appropriate antibody at 4˚C. Then, the membrane was incubated with a secondary horseradish peroxidase (HRP) conjugated antibody for 1 h at room temperature. After extensive washing, immunoreactive proteins were detected using an enhanced chemiluminescent detection system (ECL; Amersham Biosciences Corp.)

Two-hybrid system transactivation assay. The full length of PEA3 was cloned into pBIND vector (Promega Corp.), which contained the yeast GAL4 DNA binding domain upstream of a multiple cloning region to generate a fusion protein with the DNA binding domain of GAL4 (GAL4-PEA3). The pG5-Luc vector, which contained five GAL4 binding sites upstream of a minimal TATA box, and GAL4-PEA3 were cotransfected with c-Jun, TAM67 and NF-κB, respectively. The total amount of DNA was maintained at 3 μg by adding an empty vector (FLAG/pcDNA3). Luciferase activity was measured using a dual luciferase assay system (Promega Corp.).

Results

PEA3 synergizes with c-Jun to activate the HER2/neu promoter. In order to study the impact of the PEA3-dependent activation of the *HER2/neu* promoter, we constructed several deletion mutants of PEA3 expression vectors. PEA3 has a transactivation domain consisting of 42-85 representative amino acids, and a negative regulatory region at both sides. It also has the DNA binding domain called the ETS domain at the C-terminus (Fig. 1A) (6). The expression of each of the PEA3 deletion mutants was examined by Western blotting. A representative immunoblot from a single experiment is shown in Fig. 1B. A variety of genes are regulated by PEA3 and c-Jun. For example, the promoter activities of uPA (15), COX-2 (6), MMP-7 (11), and IL-8 (12) are upregulated by PEA3 and c-Jun synergistically. Experiments were therefore conducted to investigate the potential role of c-Jun in regulating the *HER2/ neu* promoter activity. The *HER2/neu* promoter plasmid was co-transfected with a combination of the indicated PEA3 constructs and c-Jun expression vector into MCF7 cells because endogenous PEA3 and c-Jun expression were very low (data not shown). As shown in Fig. 1C, PEA3 WT alone increased the *HER2/neu* promoter activity by 4-fold compared to the empty vector. However, the amino-terminal deletions extending to residues dN106, and dN278 reduced the *HER2/neu* promoter activity compared to WT. c-Jun alone increased the *HER2/neu* promoter activity by 2- to 3-fold compared to the control. On the other hand, c-Jun and PEA3 WT increased synergistically the *HER2/neu* promoter activity by up to 9-fold. Furthermore, dN32 also synergistically upregulated the *HER2/neu* promoter activity by up to 8-fold with c-Jun. In the presence of c-Jun, no increase in transcriptional activation was observable with dN278. The results suggest that PEA3 synergizes with c-Jun to activate *HER2/neu* promoter, and amino-terminus of PEA3 is important to interact with c-Jun.

HER2/neu promoter is activated by PEA3 and c-Jun. To determine if the c-Jun increases the *HER2/neu* transcriptional activity, various *HER2/neu* promoter luciferase reporter constructs were transiently transfected into MDA-MB453 cells that exhibited an increase in the expression of PEA3 and c-Jun. The promoter activity was examined by the luciferase assay, and the full-length *HER2/neu* promoter activity was set at 100%. As shown in Fig. 2, truncation of up to 286 bp of distal promoter sequences hardly influenced the promoter activity. However, further deletion of the *HER2/neu* promoter markedly reduced the activity, and the activity of the luciferase plasmid pGL3-basic was reduced to ≤10%. The region (-100/+30) includes the *Ets* binding site and the 28-bp polypurine (GGA)-polypyrimidine (TCC) mirror repeat. Together, the findings suggest that a majority of the transcription factor elements were within the first 110-bp upstream of the *HER2/neu* transcription initiation site. However, there is no AP-1 binding site in the *HER2/neu* promoter. Thus, we hypothesize that there is an important interaction between PEA3 and c-Jun for up-regulation of *HER2/neu* transcriptional activity. Thus, we examined the interaction of PEA3 with c-Jun by the two-hybrid system. We constructed a fusion protein of the GAL4 DNA-binding domain and a full-length PEA3. The specific activity of PEA3 was calculated and normalized to that of the empty vector, which was set at 1.0. The transcriptional activity of the GAL4-PEA3 fusion protein was increased by ~6-fold in the presence of exogenously expressed c-Jun. Neither TAM67 (the dominant-negative mutant of c-Jun) nor the other transcription factor, NF-κB, showed any significant effects on the transcriptional activity of the GAL4-PEA3 fusion protein (Fig. 3A). These results suggested that the transcriptional activity of PEA3 was specifically enhanced by

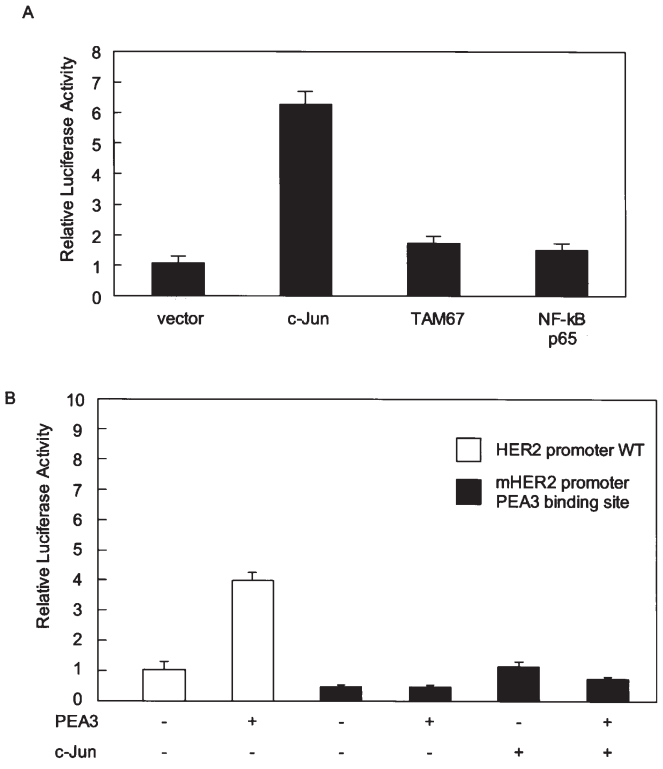

Figure 3. (A) Effect of exogenous c-Jun expression on the transactivation potential of GAL4-PEA3. The pG5-Luc plasmid and GAL4-PEA3 were cotransfected with a combination of c-Jun, TAM67 and p65 expression vectors into MCF7 cells. These were normalized to the RL-SV40 internal control. Data shown are means and SD for duplicate samples from one representative experiment. The experiment was performed three times, and similar results were obtained in each case. (B) A 3-bp inactivating mutation of PEA3 site (AGGAAG→AGCTCG) was introduced into the full length of *HER2/neu* promoter reporter constructs to create mPEA3*HER2/neu*-Luc. The mutation promoter was cotransfected with a combination of PEA3 and c-Jun expression vectors, as indicated, in parallel with the wild-type promoter.

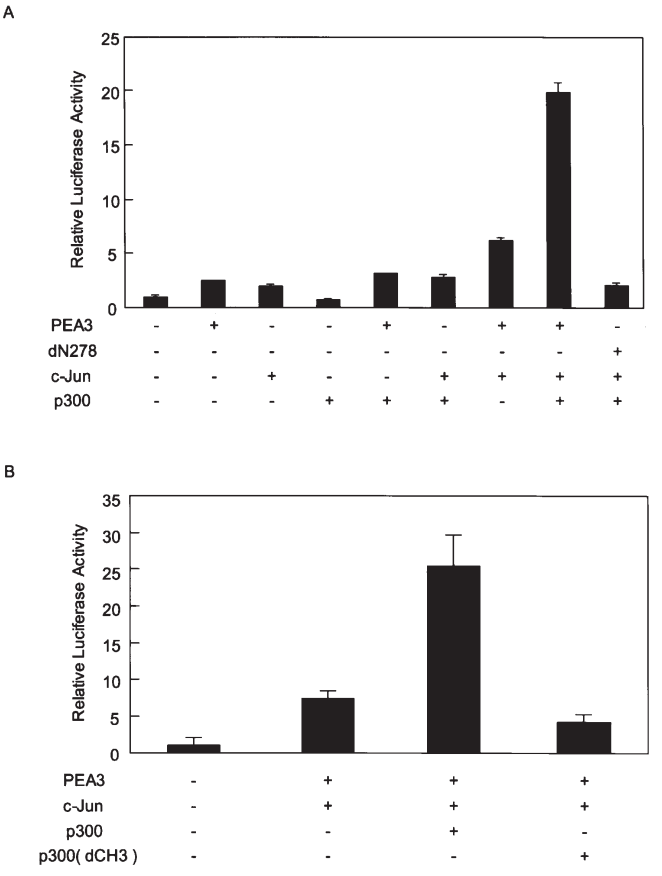

Figure 4. PEA3 and c-Jun synergy depends on the activity of p300. (A) MCF7 cells were cotransfected with *HER2/neu* promoter plasmid and combinations of PEA3, dN278, c-Jun, and p300 expression vectors, as indicated. Data shown are means and SD for duplicate samples from one representative experiment. The experiment was performed twice, and similar results were obtained in each case. (B) MCF7 cells were cotransfected with HER2/neu promoter plasmid and combinations of PEA3, c-Jun, p300 and a mutant p300 with its transactivation domain deleted (-dCH3) expression vectors, as indicated. Data shown are means and SD for duplicate samples from one representative experiment. The experiment was performed twice, and similar results were obtained in each case.

c-Jun. Furthermore, to confirm the hypothesis, we examined the activity of the *HER2/neu* promoter with a mutated PEA3-binding domain (AGGAAG to AGCTCG). When the levels of WT and mutant promoter activities were compared in the absence of PEA3, the mutant promoter activity was not induced by PEA3 (Fig. 3B). Furthermore, neither PEA3 nor c-Jun upregulated the activity of the *HER2/neu* promoter. The results suggested that PEA3 and c-Jun mediated *HER2/neu* upregulation through the PEA3 binding site on the *HER2/neu* promoter (-35/-32).

Synergistic cooperation of PEA3 with c-Jun requires activity of the transcriptional coactivator p300. We examined whether p300 increased the activity of the *HER2/neu* promoter through PEA3 and c-Jun. To test if PEA3 synergy with c-Jun was responsive to and dependent on p300 activity, PEA3 and c-Jun were coexpressed with p300 or dominant-negative p300 (p300-dCH3). p300 expression alone did not increase the activity of the *HER2/neu* promoter; however, it slightly enhanced PEA3 and c-Jun activation of the *HER2/neu* promoter (~2-fold, respectively) (Fig. 4A). p300 cooperated with both PEA3 and c-Jun activation, boosting the 7-fold activation by PEA3 and c-Jun to ~21-fold. The expression of p300-dCH3 was capable of significantly blocking PEA3 and c-Jun activation of the *HER2/neu* promoter (Fig. 4B). The findings suggest that the synergy of PEA3 and c-Jun was related to the ability to coordinately recruit p300 to the *HER2/neu* promoter.

Discussion

The major findings of the present study on *HER2/neu* gene regulation are as follows: 1) c-Jun enhances the transcriptional activity of PEA3, 2) the transcriptional induction of *HER2/neu* is mainly regulated synergistically by PEA3 and c-Jun via the *Ets* binding site on the *HER2/neu* promoter 3) coactivator p300 increases the activity of the *HER2/neu* promoter through PEA3 and c-Jun.

DNA-protein and protein-protein interactions often play major roles in the targeting of ETS-domain proteins to specific promoters (28). Because PEA3 and c-Jun increase synergistically the activity of various promoters, we analyzed the interaction of PEA3 and c-Jun on the *HER2/neu* promoter. To our knowledge, this is the first study showing that PEA3 and c-Jun increase synergistically the *HER2/neu* promoter

activity. In some cases, transcription factors acting on the AP1 site cooperate with other transcription factors to achieve efficient gene activation (29). To assess PEA3 and c-Jun regulation of *HER2/neu* gene transcription, we performed cotransfection experiments of a wild-type *HER2/neu* promoter luciferase reporter construct with the expression vectors for c-Jun or PEA3 gene, and found that PEA3 had moderate coactivator function. However, the coexpression of c-Jun with PEA3 enhanced the activity of the *HER2/neu* promoter by 9- to 10-fold. The involvement of closely spaced PEA3 and AP-1 elements has been reported for several inducible genes including uPA (15), COX-2 (6), MMP-7 (11), keratin 16 (30), tumor necrosis factor-α (31) and IL-8 (12). However, we found that the deletion construct -110/+30 *HER2/neu* promoter-Luc still exhibited an increase in the *HER2/neu* promoter activity in MDA-MB453 cells. Thus, our study suggests that the PEA3 binding site (-35/-32) may be the most important site of the *HER2/neu* promoter for PEA3 and c-Jun. As shown in Fig. 3B, the mutation of the PEA3 site construct was never upregulated by PEA3 and c-Jun. Moreover, dN278, as the dominant-negative function of PEA3, clearly inhibited the activation of the *HER2/neu* promoter even if c-Jun was overexpressed. Thus, the PEA3 binding site within the *HER2/neu* promoter was shown to be important for the upregulation of *HER2/neu* gene transcription (19,20).

Our results suggest that c-Jun is able to activate the *HER2/neu* promoter by interaction with the transcriptional activator PEA3. We surmised that PEA3 and c-Jun play a functional role in the expression of the *HER2/neu* gene through an interaction between these two transcription factors. Using a two-hybrid system, we showed that c-Jun enhances the activity of PEA3, and the dominant-negative mutant of c-Jun (TAM67) cannot increase the activity of PEA3. It has been suggested that ERM, a member of the PEA3 family, was enhanced by c-Jun (32). Thus, PEA3 and c-Jun appear to interact with each other and increase the activity of the *HER2/neu* gene synergistically. However, as there are many transcription factors that are related to *HER2/ neu* regulation, further study is required to distinguish between these interactions.

The acetylation of amino acids is a common means to modify their biological activity and, thus, altering protein-protein interaction, DNA recognition, and protein stability. Several experiments have suggested the importance of histone acetylation in the transcription of a variety of genes. p300 possesses intrinsic histone acetyltransferase activity, works as a transcriptional coactivator, and cooperates with multiple transcriptional factors. In this study, the interplay of PEA3, c-Jun and p300 in the transcriptional regulation of the *HER2/neu* gene in MCF7 cells was elucidated as well. The coactivator p300 was hypothesized to interact with PEA3 and c-Jun, thereby activating the *HER2/neu* promoter activity. Furthermore, a p300-dCH3 construct, which is the deleted CH3 domain of p300, blocked the activity of the *HER2/neu* promoter induced by PEA3 and c-Jun. The N-terminal transactivation domain of c-Jun interacts with the KIX and CH3 domain of p300 (33). It has also been reported that PEA3 and p300 interact to activate the COX-2 promoter (5). PEA3 is co-immunoprecipitated with p300 and requires p300 to transactivate the COX-2 promoter. Furthermore, p300 is involved in the transcriptional regulation of the *HER2/neu* gene and serves as a target of E1A to repress *HER2/neu* (34). Thus, one potential mechanism for this collaboration is that p300 may serve as a bridge among c-Jun, PEA3, and basic transcription machinery to modulate *HER2/neu* gene transcription. The formation of a ternary complex consisting of PEA3, c-Jun and p300 may be instrumental for the dysregulation of many genes and thereby provide one mechanism leading to cell transformation.

Such *Ets* transcription factors as Ets-1 and PEA3 may be the candidate molecular targets of selective cancer therapy because they play important roles in the maintenance of the transformed phenotypes of tumor cells. Several tyrosine kinase inhibitors, such as Glivec and Herceptin, have been developed for molecular target therapy in cancer. However, it appears difficult to develop drugs that directly block the action of *Ets* transcription factors. Accordingly, the following have been developed experimentally: 1) dominant-negative mutants, 2) antisense oligonucleotides, and 3) RNA interference. Considering that some *Ets* transcription factors regulate growth-, apoptosis-, angiogenesis-, invasion- and metastasis-related genes in tumor cells, those transcription factors may serve as the molecular targets of cancer gene therapy. A molecular targeting therapy against *Ets* transcription factors may be a novel approach to selective cancer treatment in the near future. Extensive preclinical studies have shown that these signaling cascades regulate multiple cellular processes, such as proliferation, differentiation, survival and transformation. As the targeted agents will be used in clinical trials, it will be important to have a clear understanding of the *HER2/neu*-dependent pathways and their patterns of expression and activation. The results of the present study provide novel information on the regulatory mechanisms of *HER2/neu* gene expression. Further investigation along these lines is required to clarify the physiology and pathophysiology of *HER2/neu* gene expression in health and disease, possibly resulting in specific therapeutic interventions for various diseases.

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