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The effects of the normal and oncogenic forms of the neu tyrosine kinase, and the corresponding forms of an immunoglobulin E receptor/neu tyrosine kinase fusion protein, on *Xenopus* oocyte maturation

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In this work, we have used *Xenopus* oocyte maturation as a read-out for examining the ability of the neu tyrosine kinase ($p185^{neu}$) to participate with the epidermal growth factor (EGF) receptor in a common signal transduction pathway. We find that unlike the case for the EGF receptor, which elicits EGF-dependent maturation of these oocytes as reflected by their germinal vesicle breakdown (GVBD), neither the normal neu tyrosine kinase ($p185^{val664}$) nor the oncogenic form of neu ($p185^{glu664}$) are able to effectively trigger this maturation event. However, expression of $p185^{glu664}$ causes a specific and significant promotion of the progesterone-induced GVBD, reducing the half-time for this maturation even from ~9 h to ~5 h. Stimulation of the progesterone-induced GVBD did not occur following the expression of a kinase-deficient $p185^{neu}$ protein (in which a lysine residue at position 758 was changed to alanine). Essentially identical results were obtained when the mRNAs coding for fusion proteins comprised of the extracellular domain of the receptor for immunoglobulin E (IgE), and the membrane-spanning and tyrosine kinase domains of normal or oncogenic $p185^{neu}$ (designated IgER/$p185^{val664}$ and IgER/$p185^{glu664}$, respectively), were injected into oocytes. Antigen-induced crosslinking of IgER/$p185^{val664}$ proteins expressed in oocytes caused a reduction in the half-time for the progesterone-stimulated GVBD from ~9 h to ~7 h. Thus, the aggregation of the membrane-spanning and/or tyrosine kinase domains of $p185^{val664}$ partially mimics the effects of the oncogenic forms of $p185^{neu}$. Overall, the results of these studies suggest that the activation of the $p185^{neu}$ tyrosine kinase by a point mutation within its membrane-spanning helix, or an aggregation event, can result in the facilitation of oocyte maturation events that are elicited by other factors (e.g. progesterone). However, the activated $p185^{neu}$ tyrosine kinases are not able to mimic the EGF-stimulated EGF receptor tyrosine kinase in triggering oocyte maturation, which suggests that the EGF receptor and the $p185^{neu}$ tyrosine kinase do not input into identical signal transduction pathways in these cells.

Immunoglobulin E; Neu; Oocyte; Oncogene; Tyrosine kinase

1. INTRODUCTION

One subclass of the superfamily of growth factor receptor/tyrosine kinases (i.e. designated as subclass 1) [1], which includes the epidermal growth factor (EGF) receptor, the $p185^{neu}$ tyrosine kinase (also referred to as erbB-2 or Her2), and the recently identified erbB-3, has received a great deal of attention because of the suspected involvement of its members in human cancers [2,3]. In the case of the neu/erbB-2 and erbB-3 proteins, the mechanisms underlying the activation of their tyrosine kinase activities are of interest, particularly because specific ligands (growth factors) for these proteins have yet to be definitively identified. Interestingly, one specific mode of activation of the rat $p185^{neu}$ tyrosine kinase occurs as an outcome of a single point mutation which converts a valine residue at position 664 to a glutamic acid. This point mutation is sufficient to elicit oncogenic activity [4], most likely as a result of an increased tyrosine kinase activity [5–8].

A second possible mode of activation of $p185^{neu}$ is through a tyrosine kinase cascade. This possibility is based on a number of reports which have demonstrated that the EGF receptor is able to interact with the $p185^{neu}$ tyrosine kinase [9–13]. The existence of such a cascade would imply that the EGF receptor and $p185^{neu}$ participate in a common signaling pathway. However, studies in an interleukin-3-dependent hematopoietic cell line [14] have shown that while the EGF receptor could efficiently input into the mitogenic pathways of these cells, different forms of neu/erbB-2 could not substitute for the EGF-activated EGF receptor in this system. These results then argue that the EGF receptor and $p185^{neu}$ in fact participate in distinct signal transduction pathways.

In the present study, we have used the *Xenopus* oocyte system to further address this issue. Opresko and Wiley [15] first demonstrated that the expression of the EGF receptor tyrosine kinase in oocytes resulted in the resumption of meiosis as reflected by an EGF-dependent stimulation of germinal vesicle breakdown (GVBD). In this paper, we have examined whether the translation of the mRNA for the normal or the oncogenic $p185^{neu}$

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tyrosine kinases was able to mimic the EGF receptor and elicit a stimulation of GVBD in oocytes. In addition, we have examined whether the mRNA coding for fusion proteins that contain the extracellular domain of the immunoglobulin E (IgE) receptor [16], and the membrane-spanning helix and tyrosine kinase domain of either the normal or oncogenic forms of $p185^{neu}$, can spontaneously trigger oocyte maturation or modulate maturation triggered by other agents (i.e. progesterone).

2. MATERIALS AND METHODS

2.1. *Oocyte isolation and culture*

Xenopus laevis oocytes were dissociated by treatment with collagenase (5 mg/ml, Boehringer-Mannheim) in Barth's buffer (88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 2.4 mM $NaHCO_3$, 5 mM Tris-HCl, pH 7.4) at 16°C for 5–7 h. The oocytes were injected with a model NA-1 microinjector (Sutter Instrument Co., San Rafael, CA) within 24 h of isolation with 50 nl of RNA (per oocyte) or with vehicle (water).

2.2. *Preparation of chimeric DNA constructs*

Constructs containing rat neu DNA (Glu^{664}, Val^{664}, and Val^{664}/Ala^{758}) in a pSV2-derived vector were generously provided by Dr. David Stern (Yale School of Medicine). The vector containing the human IgE receptor α-subunit (Fc-GEM) was a kind gift from Dr. J.-P. Kinet (NIADD, Bethesda, MD). The SP6-driven EGF receptor construct pLOLB, was kindly provided by Dr. H.S. Wiley (Univ. of Utah Med. Ctr). Blunt-ended *Nde*I–*Sal*I fragments of the normal and oncogenic neu constructs were cloned into a blunted *Sal*I site in pBluescript-, generating pNeuN and pNeuT, respectively. A *Sca*I fragment of the pSVNeu (Ala^{758}) construct was ligated to a fragment derived by partial *Sca*I digestion of pNeuN and pNeuT generating pN(A758) and pT(A758), respectively. The polymerase chain reaction (PCR) was used to synthesize a fragment of Fc-GEM, which precisely encoded the extracellular domain of the IgE-receptor from the unique upstream *Hin*dIII site to a novel 3′ linker that replaces regions downstream of the first putative transmembrane residue of the IgE receptor α-subunit with 18 bases encoding the transmembrane sequence of the neu gene. Another fragment was synthesized by PCR, upon the pNeuN and pNeuT construct templates, to contain as a 5′ boundary 18 bases of sequence corresponding to the extracellular juxta-membrane residues of the IgE receptor α-subunit followed by 18 bases of sequence corresponding to the transmembrane region of the neu gene product. The 3′ boundary of these fragments was defined by a linker synthesized complementary to a portion of the coding strand of the neu gene including a unique *Bsp*EI site. The PCR-derived fragments were used in an additional PCR reaction with only the 5′ Fc-GEM primer and the 3′ pSVNeu primer to generate a hybrid 650 bp fragment that encodes the region bearing the entire extracellular domain of the IgE receptor α-subunit fused in-frame to the transmembrane region of $p185^{Neu}$. This fragment was digested with *Hin*dIII and *Bsp*EI and ligated to appropriately digested pNeuN, pNeuT, PN(A758), and pT(A758) vectors.

3. RESULTS

It has been reported that the expression of the human EGF receptor in *Xenopus* oocytes, following the injection of the mRNA of the receptor, resulted in the stimulation of meiotic maturation [15]. Fig. 1 shows the results from a similar experiment where the mRNA for the EGF receptor was generated using the pOBER vector (which enables the synthesis of ≥ 10^6 receptors per cell), as originally described by Opresko and Wiley [15]. While the kinetics of the progesterone-induced GVBD differ due to varying culture conditions, the addition of nanomolar concentrations of EGF to these oocytes caused GVBD about twice as fast as progesterone. Typically, we observed an obvious change in the morphology of the oocytes 12–24 h following the addition of EGF, ultimately resulting in oocyte death. This cytotoxic effect of EGF was eliminated by the nominal removal of Ca^{2+} from the medium, consistent with the suggestion of Opresko and Wiley that the EGF-induced oocyte degeneration was the outcome of EGF-induced changes in the levels of intracellular Ca^{2+} [15].

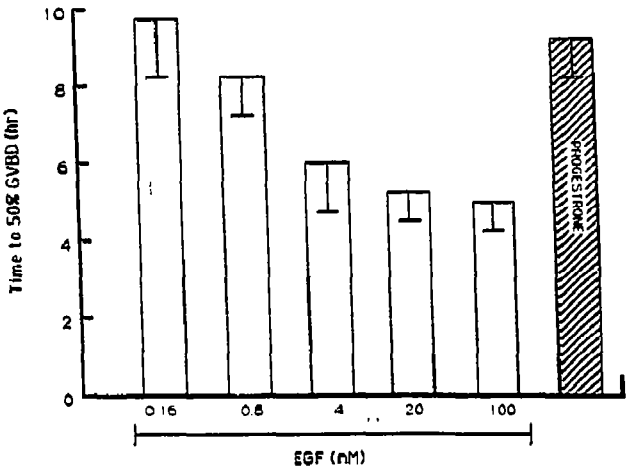

Fig. 1. EGF-stimulated GVBD in oocytes expressing message for EGF receptor. 48 h following injection with 50–100 ng of EGF receptor-encoding mRNA, oocytes were exposed to various doses of EGF. Times to the onset of GVBD as evidenced by the appearance of a white spot on the animal pole are shown. Control oocytes were exposed to 5 μg/ml progesterone. Error bars are representative of data from two experiments with each data point representing the average of approximately 20 oocytes.

We have compared the effects of different forms of the $p185^{neu}$ tyrosine kinase (Fig. 2) on oocyte maturation with those elicited by the EGF receptor. Two of these forms represented normal $p185^{neu}$ and an oncogenic form in which the valine at position 664 was changed to a glutamic acid. These are designated as $p185^{val664}$ and $p185^{glu664}$, respectively. Two other constructs coded for fusion proteins comprised of the extracellular domain of the IgE receptor and the membrane-spanning helix and cytoplasmic domain of the normal, or oncogenic, $p185^{neu}$ tyrosine kinase. These two fusion proteins are designated IgER/$p185^{val664}$ and IgER/ $p185^{glu664}$, respectively. An advantage of the IgER/ $p185^{neu}$ fusion proteins was that the levels of expression of these proteins in oocytes could be determined by measuring the binding of labeled IgE to the fusion proteins [15]. This differs from the case of $p185^{neu}$ for which the quantitation of protein expression through ligand binding assays has not been possible

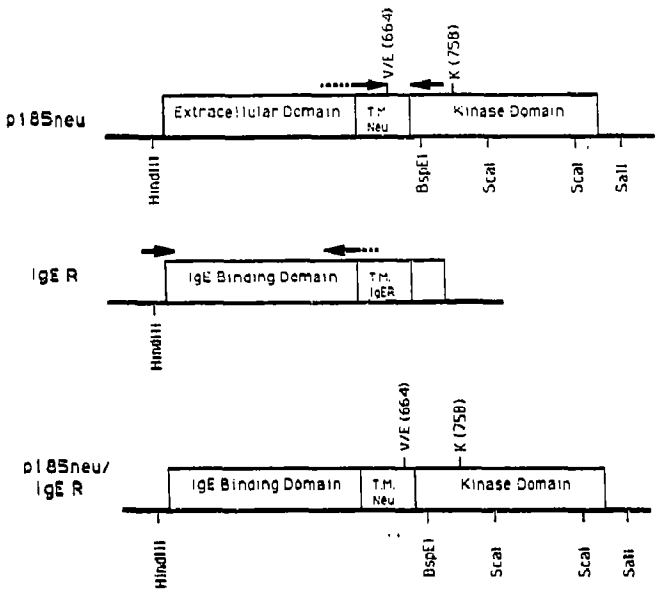

Fig. 2. Schematic representation of restriction sites and primary structure of the parent $p185^{neu}$ and chimeric IgE receptor/$p185^{neu}$ constructs. T.M. denotes the transmembrane domain. V/E(664) represents the normal and activating residues in the transmembrane segment of $p185^{neu}$. The orientation and complementarity of primers used in the hybrid-generating PCR reaction are also shown. Non-complementary regions in each primer are denoted by dashed lines.

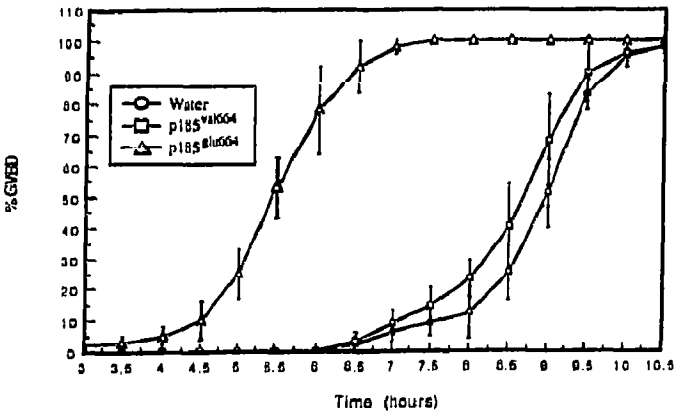

Fig. 3. Expression of $p185^{val664}$ enhances the rate of progesterone-induced oocyte maturation. Oocytes were injected with 50–75 ng of mRNA as described in Materials and Methods. 48 h following injection sample- and water-injected control oocyte pools (approx. 50–60 oocytes each) were exposed to 5 μg/ml progesterone. The time to onset of GVBD is shown.

since $p185^{neu}$-specific ligands (e.g. growth factors) have not been definitively identified (although see ref. [17]). In addition, since protocols for crosslinking IgE receptor extracellular domains and for generating IgE receptor oligomers have been established [18], the IgER/$p185^{neu}$ chimeras provided a means for examining the possible involvement of protein–protein interactions (aggregation) in the activation of $p185^{neu}$ tyrosine kinase activity.

Unlike the case for the EGF receptor, we did not find any conditions where either the normal neu tyrosine kinase ($p185^{val664}$) alone, or the oncogenic form of neu($p185^{glu664}$) alone, was able to elicit GVBD in the oocytes. However, we did observe that after a period of 2–4 days, some percentage of the oocytes (~30–40%) that were injected with the mRNA for $p185^{glu664}$ (and to a much lesser extent when injected with the mRNA for $p185^{neu}$) began to show an abnormal morphology and ultimately died. The cell death elicited by the $p185^{neu}$ tyrosine kinases could be inhibited by >80% by depleting the oocytes of extracellular Ca^{2+} (data not shown), similar to the case for the EGF receptor.

Progesterone potently elicits GVBD, as has been documented by a number of laboratories [cf. 19], and we find that the oncogenic form of the neu tyrosine kinase ($p185^{glu664}$) can accelerate this response (see Fig. 3 and Table I). Following the injection of the message for the $p185^{glu664}$ into oocytes, there was a significant shift in the half time for the progesterone-induced GVBD from ~9–9.5 h (i.e. for control oocytes which were injected with water) to ~5 h. The promotion of the progesterone-induced maturation was specifically elicited by $p185^{glu664}$, with the injection of the message for $p185^{val664}$ resulting in little or no enhancement (i.e. typically <2%; see Fig. 3 and Table I, entries 1–3). In addition, when the lysine at position 758 of the $p185^{glu664}$ protein was changed to an alanine residue, the resultant kinase-defective neu was unable to promote the progesterone-induced maturation event (see Table I, entries 4 and 5).

Fig. 4 presents the results of experiments where the IgE receptor/$p185^{neu}$ chimeras were expressed in oocytes. Based on assays measuring the binding of ^{125}I-IgE to IgER/$p185^{neu}$ fusion proteins, we estimated that ~5×10^6 IgER/$p185^{neu}$ chimeras were expressed per oocyte in these experiments. As was observed with the $p185^{glu664}$, the IgER/$p185^{glu664}$ chimera caused a signifi-

Table I

Effects of microinjected mRNAs encoding the $p185^{neu}$ tyrosine kinase on progesterone[1]-stimulated oocyte maturation

Injections	Time to 50% GVBD[2] (hours)
1. Water injected	9.0 ± 0.2
2. $p185^{val664}$ mRNA	8.8 ± 0.2
3. $p185^{glu664}$ mRNA	5.6 ± 0.5
4. $p185^{val664ala758}$ (-kinase) mRNA	8.7 ± 0.3
5. $p185^{glu664ala758}$ (-kinase) mRNA	8.8 ± 0.3
6. IgER/$p185^{val664}$ mRNA	8.6 ± 0.3
7. IgER/$p185^{glu664}$ mRNA	5.5 ± 0.4
8. Y13-259 (150 ng/oocyte)	8.4 ± 0.2
9. IgER/$p185^{val664}$ mRNA + Y13-259 (150 ng/oocyte)	8.4 ± 0.4
10. IgER/$p185^{glu664}$ mRNA + Y13-259 (150 ng/oocyte)	5.4 ± 0.5

[1] Progesterone was used at 5 μg/ml.
[2] Standard deviations are based on two experiments with each data point representing the average of approximately 30 oocytes.

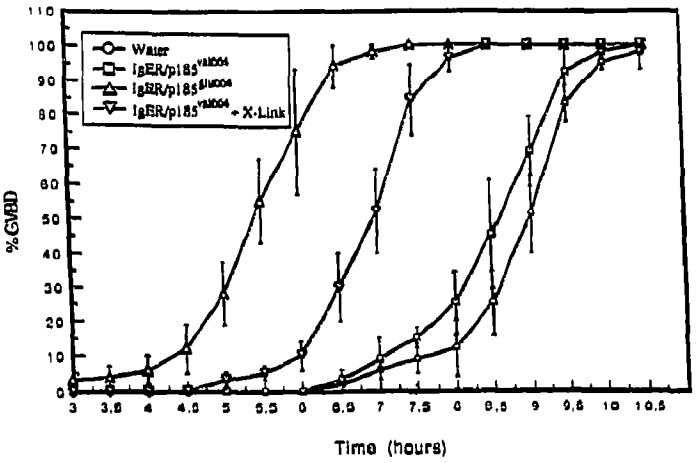

Fig. 4. Effect of chimeric proteins on progesterone-induced oocyte maturation. Injected oocyte pools (50–75 ng mRNA/oocyte, 50–60 oocytes/pool) were exposed to monomeric anti-DNP mouse IgE (10 μg/ml) for 48 h post-injection. Subsequently, progesterone (5 μg/ml) and cross-linking agent (DNP_{24}-BSA, 1 μg/ml) were simultaneously added to the washed oocytes. The onset of GVBD was recorded as described.

cant increase in the rate of progesterone-stimulated GVBD, whereas its non-mutated counterpart, IgER/$p185^{val664}$, caused only a minor reduction in the half life for GVBD relative to the rate measured following the injection of water. Here, again the substitution of an alanine residue at position 758 for a lysine residue eliminates the ability of the IgER/$p185^{glu664}$ chimera to promote the progesterone-stimulated GVBD event (data not shown). Taken together, these data indicate that the extracellular domain of $p185^{neu}$ is not essential for the ability of the oncogenic form of $p185^{neu}$ (i.e. $p185^{glu664}$) to elicit a biological response, whereas a viable tyrosine kinase activity is essential for the stimulated GVBD by the neu proteins.

It has been well documented that the binding of IgE to the extracellular domain of the IgE receptor, followed by the antigen-induced crosslinking of IgE-bound IgE receptor complexes, results in an activation of the receptor signaling pathways [18]. The results presented in Fig. 4 show that the crosslinking of the IgER/$p185^{val664}$ chimera, by the addition of 1 μg/ml dinitrophenyl-conjugated BSA (DNP_{24}-BSA), resulted in an increase in the rate of the progesterone-stimulated GVBD, relative to the rate observed with IgER/$p185^{val664}$ in the absence of crosslinking. The half time for progesterone-induced oocyte maturation was reduced from ~9 h to ~7 h, which indicates that the crosslinked IgER/$p185^{val664}$ chimeras were about 50% as effective as the oncogenic form of $p185^{neu}$ in eliciting this specific biological response. The crosslinking of IgER/$p185^{glu664}$ chimeras was not observed to cause any significant change in the half-time for GVBD relative to that measured in the absence of crosslinking (data not shown).

4. DISCUSSION

The results described here suggest that $p185^{neu}$ does not fully mimic the EGF receptor in its ability to influence cell cycle events in oocytes. In fact the EGF receptor appears to be one of the most effective agents known with regard to triggering oocyte maturation (where, for example, in our hands it clearly surpasses the ability of insulin to elicit a maturation event and at least equals the effectiveness of progesterone). However, we find that even the oncogenic form of $p185^{neu}$ ($p185^{glu664}$) shows little or no ability to stimulate spontaneous GVBD in oocytes. Thus these findings, together with the studies by DiFiore et al., in interleukin-3-dependent 32D hematopoietic cells [14], lead to the conclusion that the EGF receptor and neu tyrosine kinases do not share identical cellular signaling pathways.

The oncogenic form of $p185^{neu}$ does significantly enhance the ability of progesterone to stimulate oocyte maturation. The molecular basis underlying the observation that only the oncogenic form of $p185^{neu}$ is capable of eliciting this enhancement is currently unknown. However, various groups have suggested that the single mutation within the membrane-spanning helix of $p185^{neu}$ (at position 664) results in an increased tyrosine kinase activity [5–8], thus suggesting that a tyrosine phosphorylation event(s) is an important component of the enhancement of the progesterone-stimulated GVBD. The fact that this enhancement is not observed with a kinase-defective form of $p185^{glu664}$ is consistent with such a notion.

It is interesting that the $p185^{glu664}$ and IgER/$p185^{glu664}$ proteins appear to have essentially identical abilities to promote the progesterone-stimulated maturation event. This indicates that the extracellular domain of $p185^{neu}$ is not an essential component of the biological activities elicited by the oncogenic form of $p185^{neu}$. However, the aggregation of the IgER extracellular domains of the IgER/$p185^{neu}$ chimera does appear to significantly increase the ability of a normal $p185^{neu}$ protein (i.e. containing valine at position 664) to enhance the progesterone-stimulated GVBD. This is consistent with an earlier suggestion [20] that the aggregation of $p185^{neu}$ causes an elevation of $p185^{neu}$ tyrosine kinase activity. Nonetheless, this aggregation event does not result in a facilitation of progesterone-induced GVBD comparable to that observed upon expression of $p185^{glu664}$. Possible explanations for this observation may involve an incomplete (or improper) aggregation of $p185^{neu}$ following the antigen-induced crosslinking of the IgER extracellular domains, or it may mean that the aggregation of $p185^{val664}$ does not fully mimic the tertiary conformation of $p185^{glu664}$.

Previous studies have suggested that the $p21^{ras}$ GTP-binding proteins are downstream components of the signaling pathways of growth factor receptor/tyrosine kinases [21], and the microinjection of oncogenic forms of $p21^{ras}$ can elicit oocyte maturation [22]. Thus far, we have not found the injection of the neutralizing $p21^{ras}$ antibody Y13-259 (150 ng/oocyte) to have any effect on the ability of $p185^{glu664}$ to promote the progesterone-induced maturation (see Table I, entries 8–10) or to influence the ability of the EGF receptor to elicit EGF-dependent oocyte maturation (data not shown). Future studies, aimed at comparing the effects of the $p21^{ras}$ protein on oocyte maturation with those of the EGF receptor and $p185^{neu}$, should provide important information regarding the possible role of this GTP-binding protein in the signaling pathways of these tyrosine kinases.

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