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N-terminal to the si	te of phosphorylation.	We have also demon	strated the	
feasibility of using libraries for Neu su	a novel solid-phase k bstrates. However, th	inase assay to scree e Neu protein that w	n <u>E. coll</u> expression e isolated is very	
unstable, and we hav	e had problems produci	ng large quantities	of Neu. Thus, we	
are currently workin	g on two new expression ication, then move to	n systems. We will the library screenin	optimize Neu g procedures. The	
final phase of the p	roject will be to use	this information to	generate inhibitors.	
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#### **INTRODUCTION**

Constitutive activation of signaling pathways involving tyrosine phosphorylation is believed to play a crucial role in human breast cancer. The receptor tyrosine kinase p185<sup>HER2/neu</sup> (Neu) is amplified in 20 to 30% of human primary breast cancers, and expression of Neu in tumor tissues is correlated with poor clinical prognosis (1). Transfection of NIH3T3 cells with the *neu* oncogene results in malignant transformation (2), and studies in transgenic mice that overexpress Neu indicate that breast tissue is particularly susceptible to the transforming properties of the *neu* oncogene (3). Antibodies that bind to Neu can inhibit the growth of cells overexpressing Neu (4), suggesting that Neu may be a clinically useful target.

Neu is a 185 kilodalton protein that is structurally very similar to the EGF receptor: it has a cysteine-rich extracellular domain, a membrane-spanning region, and an intracellular tyrosine kinase domain (5). The mechanism by which Neu transmits its mitogenic signal is not completely clear, but the tyrosine kinase activity of the receptor plays a crucial role (1). Overexpression of Neu may result in constitutive activation of signalling pathways involved in proliferation of normal cells. For example, the nonreceptor tyrosine kinase pp60<sup>c-src</sup> (c-Src, the product of the *c-src* proto-oncogene) associates with activated Neu in human breast carcinoma cell lines and the tyrosine kinase activity of c-Src is elevated (6). The identities of the *in vivo* substrates for Neu are unknown; in breast cancer cells, Neu may phosphorylate and activate Src, or may phosphorylate other membrane-bound substrates. The substrate specificity of the Neu tyrosine kinase has not previously been defined. The identification of substrates for Neu and the study of the enzyme's substrate specificity offers a therapeutic approach to anticancer drug design based on selective interference with signalling pathways emanating from this receptor tyrosine kinase.

We hypothesize that Neu contributes to malignant growth of breast cancer cells by phosphorylating plasma membrane proteins. The broad goal of this project is to obtain information about the substrate specificity of the Neu tyrosine kinase. This information will provide a clearer understanding of how this enzyme subverts the normal signalling pathways in cells to cause neoplastic growth. In the second phase of the project, we will attempt to design molecules that specifically interfere with the action of Neu tyrosine kinase *in vivo* and *in vitro*. The results of these investigations may suggest avenues for design of specific anticancer agents.

The specific objectives of our project are:

*Objective 1.* We will isolate the Neu tyrosine kinase from cultured breast cancer cells and study its specificity using combinatorial peptide libraries.

*Objective* 2. We will also study the specificity of Neu with the solid-phase tyrosine kinase assay recently developed in our laboratory. Specificity will be screened using an *E. coli* expression library with glutathione S-transferase as the fusion partner. Recognition sites for Neu within the library will be identified by the solid-phase kinase assay, followed by DNA sequencing of positive clones.

*Objective 3.* Nonphosphorylatable tyrosine mimetics will be introduced into peptide sequences obtained from Objectives 1 and 2. These compounds will be tested as inhibitors of the Neu tyrosine kinase *in vitro*. Results will be compared for representatives of other classes of tyrosine kinases.

*Objective 4.* Active compounds from Objective 3 will be truncated stepwise from the N- and C-termini to determine the minimum peptide length necessary for specific inhibition of Neu. The resulting peptides will be tested as inhibitors of the proliferation of a breast cancer cell line, SKBR3, using a tritiated thymidine incorporation assay.

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#### **BODY OF REPORT**

#### Objective 1. Studying Neu specificity with peptide libraries.

Task 1. Purifying Neu from cultured SKBR3 breast cancer cells. In our initial experiments, we successfully isolated Neu from a cultured breast cancer cell line that overexpresses Neu, SKBR3 (obtained from American Type Culture Collection). To isolate the Neu kinase, we incubated cell lysates from logarithmic-phase SKBR3 cells with immobilized Src SH2-SH3 domains, as described (6). We prepared a fusion protein containing the Src SH2 and SH3 domains fused to glutathione S-transferase (7). This fusion protein was then coupled to glutathione-agarose. After SKBR3 cell lysates were passed over the column, the samples were washed extensively and Neu was eluted by treatment with reduced glutathione. This strategy allows recovery of active tyrosine kinases without a denaturing step during purification (8). The identity of the Neu tyrosine kinase was confirmed using SDS-PAGE with immunodetection by anti-Neu antibody cAb-1 (Oncogene Science), as well as by anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology). Representative immunoblots are shown in Figure 1. The yield of purified Neu was reasonable (Fig. 1A, lane 1; Fig. 1B, lane 1). We confirmed that the purified enzyme was active by measuring both enzyme autophosphorylation and phosphorylation of the synthetic peptide Arg-Arg-Leu-Ile-Glu-Asp-Ala-Ile-Tyr-Ala-Arg-Gly. However, we encountered a problem that will preclude using the SKBR3-purified Neu for the library experiments: the protein appears to be degraded rapidly. The molecular weight of Neu purified from SKBR3 cells is approximately 50kD; this corresponds roughly to the intracellular domain, but it is far too small to represent the entire Neu receptor (approximately 185 kD). When we examined the proteins in crude SKBR3 cell lysates that react with the Neu antibody, we observed a pattern of smallmolecular weight proteins, presumably due to the instability of full-length Neu (Fig. 1B, lanes 2 and 3). Thus, although we were able to isolate active Neu from these cells, we had concerns about the stability of the protein. Because our later objectives will require a high-yield, reliable source of Neu, we decided to try other systems to produce Neu (see below, under Objective 2, Task 1).

**Task 2.** Preparation of peptide libraries. We recently described the synthesis and use of combinatorial peptide libraries as probes for the substrate specificity of serine and tyrosine protein kinases (9). In this approach, amino acid positions proximal to tyrosine in a synthetic peptide are randomized, and peptides that are most highly phosphorylated by a given protein kinase are selected from the library. We have used this technique to identify determinants for substrate recognition by cAMP-dependent protein kinase and by the v-Abl tyrosine kinase (9). More recently, we used this strategy to identify determinants for substrate recognition by the PDGF receptor tyrosine kinase (10).

In our experiments, the peptide libraries are prepared using solid-phase synthesis with a modification of previous strategies (11). We carry out the synthesis of the peptide libraries are prepared by solid-phase peptide synthesis using a t-Boc protection strategy (12). At each degenerate position, the peptide-resin is divided into 20 equal parts by weight. Each part is coupled twice to a 6-fold excess of activated HOBt ester of a unique amino acid. Qualitative ninhydrin tests are performed after each individual coupling to ensure complete reaction. After coupling, all parts are recombined before proceeding to the next cycle.

Peptide libraries are designed to include two adjacent basic residues (arginine or lysine) at either the amino or carboxyl terminus. This allows for the quantification of phosphorylation by the phosphocellulose paper assay (13). A minimal length of six amino acid residues is believed to be required for recognition by tyrosine kinases (14). The method for phosphopeptide isolation described here is suitable for libraries with four or fewer degenerate positions (using all 20 common amino acids at each position). For this project, we synthesized two libraries of  $20^4$  (= 160,000) peptides in which residues both N-terminal and C-terminal to tyrosine have been randomized (Libraries 1 and 2; see below).

Library 1: Ala-Ala-X-X-X-Tyr-Ala-Ala-Arg-Arg-Gly Library 2: Ala-Ala-Tyr-X-X-X-Ala-Ala-Arg-Arg-Gly

(where **X** represents an equimolar mixture of all 20 common amino acids at a given position). Analysis of the libraries by Edman degradation, amino acid analysis, and mass spectrometry indicates that they contain all of the desired sequences in relatively uniform concentrations.

We have also entered into a collaboration with Dr. Peter Nestler of Cold Spring Harbor Laboratory to screen more highly degenerate libraries with the Neu tyrosine kinase. Dr. Nestler's laboratory has reported that combinatorial libraries may be attached to solid supports for screening (15). In the case of Neu, the bead-supported peptide library will be incubated with purified Neu and  $[\gamma^{-32}P]$ -ATP. Radiolabelled peptides attached to the solid support will be fixed in agarose gels, and located by overlaying the gel with a radiographic film (Figure 2). Beads giving radiographic signals will be recovered and respread at higher dilution. Usually after two cycles, individual beads can be identified and sequenced (15). Although this appears to be a promising method for screening Neu specificity, we will still proceed according to the original plan, using our own detection system in addition to Dr. Nestler's system. Our own detection system for phosphorylated peptides involves passage of the reaction mixture over anti-phosphotyrosine agarose to remove unphosphorylated peptides. After elution with phosphotyrosine, HPLC (on a C18 reverse-phase column) is used to separate the phosphopeptides. Peaks of absorbance eluted from the column are analyzed for radioactivity in a scintillation counter, and the sequence(s) of the peak(s) with the highest level of radioactivity is determined by amino acid analysis and gas-phase Edman degradation.

Task 3. Phosphorylation of peptides by Neu. We have carried out initial experiments using synthetic peptides to examine the substrate specificity of purified Neu. These experiments were carried out using Neu that was expressed in Sf9 cells (see below, under Objective 2, Task 1). We measured phosphorylation of peptide substrates using  $[\gamma^{-32}P]$ -ATP with the phosphocellulose paper assay (13). In these initial experiments, we examined three substrates that are known to be efficient substrates for different classes of tyrosine kinases: (1) RRLIEDAEYAARG, a good substrate for erbB kinase; (2) RRLIEDAIYAARG, a substrate for nonreceptor tyrosine kinases such as Src and Abl (9); and (3) RRLEEEEEAYG, a peptide that is a good substrate for insulin receptor kinase (16). Interestingly, Neu appears to prefer peptide 3, the peptide with a string of acidic residues N-terminal to tyrosine (Figure 3). We note also that Peptide 1 is better than Peptide 2; the only difference between these two sequences is an acidic residue (Glu) N-terminal to tyrosine in Peptide 1. Taken together, these results suggest that N-terminal acidic residues are favorable determinants for substrate recognition in Neu.

We have carried out initial experiments with Neu purified from Sf9 cell lysates on a peptide library (Library 1, sequence given above). Neu is able to phosphorylate a component (or components) of the library, as determined from the phosphocellulose paper binding assay. However, the levels of peptide phosphorylation were fairly low. We believe that this is due to the instability of the protein (see below). We are therefore placing the majority of our effort into developing a more reliable and efficient source of Neu, as described below. We will then move ahead with the library reactions on a larger scale for isolating phosphorylated sequences.

**Task 4.** Phosphorylation kinetics of individual peptides. As described above in Task 3, we have compared the phosphorylation of three peptide substrates and learned that Neu prefers acidic residues N-terminal to tyrosine (in contrast to nonreceptor tyrosine kinases such as Src). However, because we have not carried out the large-scale library reactions yet to determine

phosphorylated sequences, we have not been able to prepare individual sequences corresponding to the optimal sequences.

#### **Objective 2.** Studying Neu specificity with a solid-phase kinase assay.

**Task 1. Preparing Neu for the solid-phase kinase assay.** As described above, SKBR3 breast cancer cells appear to be unsuitable for large-scale production of Neu. We attempted to develop alternative methods for producing Neu. We obtained the cDNA for Neu and expressed the cytoplasmic domain using the baculovirus/Sf9 insect cell system. The cytoplasmic domain of Neu (583 amino acid residues) was fused to a FLAG epitope for rapid purification. We infected Sf9 cells with the Neu baculovirus and plated cells in large (175 cm<sup>2</sup>) tissue culture flasks. We harvested the cells, resuspended them in fresh growth medium, and continued the incubation in a spinner flask. We purified the protein under nondenaturing conditions by absorption to immobilized anti-FLAG antibody (Eastman Kodak), followed by washing and elution with excess FLAG peptide. The preparation of Neu that we obtained from this procedure contained two components, as assessed by SDS-PAGE with Coomassie blue staining, or by anti-Neu Western blotting (Fig. 4). However, we observed that the cytoplasmic domain of Neu is also degraded rapidly inside the Sf9 cells. The purified protein was not of the expected size (65 kD) for the entire cytoplasmic domain, and anti-Neu Western blots of the crude Sf9 cell lysates demonstrated that the protein is largely proteolyzed into smaller fragments when expressed in Sf9 cells (Fig. 4, lane 3).

We decided to monitor the production of Neu in Sf9 cells as a function of time after infection with the recombinant baculovirus. These results are shown in Fig. 5. The expressed Neu appears to be degraded whether cells are harvested 1, 2, 3, or 4 days post-infection (Fig. 5). Thus, the production of Neu in large amounts remains a problem. We are currently attempting to solve the problem in two ways: (1) by expressing full-length Neu (rather than a truncated cytoplasmic domain) in Sf9 cells using a baculovirus vector; (2) by expressing the cytoplasmic domain of Neu as a fusion protein with glutathione S-transferase in bacteria. We have carried out similar experiments with several other kinases (8,17), including the catalytic domain of the v-erbB kinase, which is very closely related to Neu. Thus, we believe this method may have the highest probability of success.

*Task 2. Probing E. coli expression library with Neu.* We have carried out initial experiments to demonstrate the feasibility of our solid-phase kinase assay for studying Neu. In this study, we chose to use Src as a substrate, because phosphorylation of Src by Neu has previously been reported by others (6). We expressed GST-Src (or GST alone) in *E. coli*. Plates containing bacterial colonies expressing Src were overlaid with nitrocellulose filters that were soaked in isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG) to induce expression of the GST fusion proteins. The filters were then removed from the plates and their bacterial colonies were lysed over chloroform. After washing, the filters (containing immobilized proteins from the expression library) were incubated with Neu purified from the Sf9 cell lysates in polyethylene bags in HEPES buffer (pH 7.5) containing MgCl<sub>2</sub>, MnCl<sub>2</sub>, and [ $\gamma$ -<sup>32</sup>P]ATP. The experiments show that Neu phosphorylates Src in this assay, but not a control of GST alone (Fig. 6). Thus, Neu is able to phosphorylate proteins from *E. coli* cell lysates when immobilized on nitrocellulose, as we observed previously for Abl and other tyrosine kinases.

We have prepared two degenerate libraries fused to GST for expression in *E. coli*. The first library, as described in the original proposal, contains 16 codons of completely randomized sequence fused to GST. We have also recently prepared another library with a fixed tyrosine residue and four degenerate codons surrounding tyrosine. This library was prepared by PCR mutagenesis, and is also fused to GST as a carrier protein. However, in order to successfully isolate Neu substrates from these libraries, we believe that we must first develop a stable, more

reliable preparation of Neu with high activity. As described above, this is our highest priority at the present time in the project.

#### FIGURE LEGENDS

<u>Figure 1</u>. Expression of Neu from SKBR3 breast cancer cells. Lysates from logarithmic SKBR3 cells were prepared as in ref. 6. Neu was purified from the lysates by affinity chromatography on immobilized SrcSH2-SH3 domains, followed by concentration on a Centriplus-30 unit (Amicon). Samples were analyzed by SDS-PAGE on a 9% gel, then electroblotted onto PVDF membrane. Detection was by Western blotting and ECL detection (Amersham). Molecular weight markers are indicated on the left of the gels. (A) anti-phosphotyrosine detection. Lane 1, purified Neu. Lanes 2-4, flow-through from affinity column. Lane 5, autophosphorylated Neu (Neu was incubated with 100  $\mu$ M ATP and 10 mM MgCl<sub>2</sub> for 1 hour). Lane 6, SKBR3 cell lysate prepared by sonication; Lane 7, SKBR3 cell lysate prepared in a French pressure cell. (B) anti-Neu detection. Lane 1, purified Neu. Lane 2, SKBR3 cell lysate prepared by sonication. Lane 3, SKBR3 cell lysate prepared in a French pressure cell. (and 4) micrograms, respectively).

<u>Figure 2</u>. Strategy for screening combinatorial libraries that are attached to solid supports. These experiments will be carried out in collaboration with Dr. Peter Nestler of Cold Spring Harbor.

<u>Figure 3</u>. Enzymatic activity of Neu purified from Sf9 cell lysates. Kinase assays were performed with 1 mM peptides using the phosphocellulose paper binding assay. (A) Screening peptides 1-3. Kinase reactions proceeded for 10 minutes at 30 °C. (B) Phosphorylation of Peptide 3 by Neu.

<u>Figure 4.</u> Anti-Neu Western blot of Neu purified from Sf9 cells. Neu was purified by anti-FLAG immunoaffinity chromatography, as described in the text. Procedures for Western blotting were similar to those described in the legend to Figure 1. Lanes 1 and 2, 2.5 and 5  $\mu$ g of purified protein, respectively. Lane 3, 20 microliters of crude lysate from Sf9 cells expressing Neu.

Figure 5. Expression of Neu cytoplasmic domain in Sf9 cells. Neu-infected cell lysates were analyzed by SDS-PAGE and anti-Neu Western blotting. Lanes 1-4 show lysates taken from cells 1-4 days after infection.

<u>Figure 6.</u> Solid-phase kinase assay using Neu purified from Sf9 cells. Bacterial cells expressing GST-Src or GST alone were overlaid with nitrocellulose filters that were soaked in isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG) to induce expression of the GST fusion proteins. The filters were then removed from the plates and their bacterial colonies were lysed over chloroform. After washing, the filters (containing immobilized proteins from the expression library) were incubated with Neu purified from the Sf9 cell lysates in polyethylene bags in HEPES buffer (pH 7.5) containing 10 mM MgCl<sub>2</sub>, 10 mM MnCl<sub>2</sub>, and 100  $\mu$ M ATP. Phosphorylated proteins were detected using anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology). A representative portion of each filter is shown.



В

Α



Figure 1

10





Peptide 1= RRLIEDAEYAARG Peptide 2 = RRLIEDAIYAARG Peptide 3 = RRLEEEEEAYG



Figure 3

Α

В

12



,



## GST-Src





GST



15

Figure 6

#### **CONCLUSIONS**

The overall goal of this project is to characterize the substrate specificity of the Neu tyrosine kinase. In the first year of this project, we have isolated the Neu protein from two sources: (1) from SKBR3 breast cancer cells; and (2) from Sf9 insect cells using a baculovirus expression vector. The Neu that we have prepared from these sources is active. We report here an initial characterization of the enzyme's substrate specificity. Neu, unlike nonreceptor tyrosine kinases, appears to prefer acidic residues N-terminal to tyrosine. We have also done initial experiments demonstrating the feasibility of using peptide libraries to study the specificity of Neu. However, we encountered a problem at this stage of the work: the protein is highly unstable, and we have had difficulty producing large quantities of Neu. We are currently working on two new expression systems to overcome this problem; we will optimize expression and purification, then move on to the library screening procedures.

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