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Biochemical characterization of the protein tyrosine kinase homology domain of the ErbB3 (HER3) receptor protein

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The putative protein tyrosine kinase domain (TKD) of the ErbB3 (HER3) receptor protein was generated as a histidine-tagged recombinant protein (hisTKD-B3) and characterized enzymologically. CD spectroscopy indicated that the hisTKD-B3 protein assumed a native conformation with a secondary structure similar to that of the epidermal growth factor (EGF) receptor TKD. However, when compared with the EGF receptor-derived protein, hisTKD-B3 exhibited negligible intrinsic protein tyrosine kinase activity. Immune complex kinase assays of full-length ErbB3 proteins also yielded no evidence of catalytic activity. A fluorescence assay previously used to characterize the nucleotide-binding properties of the EGF receptor indicated that the ErbB3 protein was unable to bind nucleotide. The hisTKD-B3 protein was subsequently found to be an excellent substrate for the EGF receptor protein tyrosine kinase, which suggested that *in vivo* phosphorylation of ErbB3 in response to EGF could be attributed to a direct cross-phosphorylation by the EGF receptor protein tyrosine kinase.

INTRODUCTION

Discovered by molecular cloning [1,2], the *ErbB3* gene encodes a member of the ErbB subfamily of receptor protein tyrosine kinases [3]. Like the prototypical epidermal growth factor (EGF) receptor, the ErbB3 protein is predicted to consist of an extracellular ligand-binding domain, a transmembrane domain, an intracellular protein tyrosine kinase domain (TKD) and a C-terminal phosphorylation domain. Despite its structural similarity to other ErbB family receptors (EGF receptor, ErbB2/Neu, ErbB4), the presence of protein tyrosine kinase activity in ErbB3 has been questioned [2], as the deduced amino acid sequence of the protein shows three substitutions for residues invariantly conserved in all protein tyrosine kinases with known sequence [4]. Efforts to resolve this question have led to conflicting results. Two groups have detected ligand-stimulated protein tyrosine kinase activity in a chimaeric EGF receptor/ErbB3 protein, and concluded that the ErbB3 cytosolic domain possesses intrinsic catalytic activity [5,6]. However, a third group found negligible protein kinase activity in a recombinant bovine ErbB3 protein [7].

Recently, the ErbB3 protein has been shown to bind EGF-related polypeptides in the neuregulin (heregulin) family [8–10]. In cultured cells expressing ErbB3, the protein has been seen to be phosphorylated on tyrosine residues in response to EGF or neuregulin [11–13]. As this phosphorylation is dependent on the co-expression of either the EGF receptor or ErbB2 [14–21], it has been considered that the ErbB3 protein may be a physiological substrate for the protein tyrosine kinase activities of the EGF receptor and ErbB2. Indeed it appears that the ErbB3 protein may form receptor heterodimers with either the EGF receptor or ErbB2 protein (reviewed in [22–24]). The role of any intrinsic protein tyrosine kinase activity of ErbB3 in the phosphorylation of ErbB3 and its associated ErbB family members within the context of receptor heterodimers remains unclear.

In order to assess the catalytic potential of ErbB3, the cytosolic domain of the protein and that of the well-characterized EGF receptor were generated by use of the baculovirus/insect cell expression system. The purified recombinant proteins were characterized by CD spectroscopy, protein tyrosine kinase activity assays and a recently described nucleotide-binding assay [25]. The recombinant ErbB3 protein was seen to be devoid of intrinsic protein tyrosine kinase activity, and indeed appeared unable to bind nucleotide. The ErbB3 cytosolic domain was subsequently found to be an excellent substrate for the EGF receptor protein tyrosine kinase. Together these results indicated that the observed phosphorylation of ErbB3 in the cellular context might be effected by the protein tyrosine kinase activities of other ErbB family members.

EXPERIMENTAL

Cell lines and reagents

All cell lines were purchased from American Type Culture Collection and cultured as recommended. 2′(3′)-*O*-(2,4,6-Trinitrophenyl)adenosine 5′-triphosphate (TNP-ATP) was obtained from Molecular Probes. [γ-^{32}P]ATP ($\sim$ 3000 Ci/mmol) was supplied by Dupont–New England Nuclear. ErbB3-specific (2F12) and EGF-receptor-specific (LA1) monoclonal antibodies were purchased from NeoMarkers and Upstate Biotechnology respectively. Phosphotyrosine-specific monoclonal antibody (PY20) was obtained from Leinco Technologies. Horseradish

Abbreviations used: EGF, epidermal growth factor; TNP-ATP, 2′(3′)-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate; TKD, protein tyrosine kinase domain; hisTKD61, C-terminally complete EGF receptor cytosolic domain protein; hisTKD38, C-terminally truncated EGF receptor cytosolic domain; hisTKD-B3, ErbB3 cytosolic domain protein.

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peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents were purchased from Amersham. The fusion protein GST–TK7 [26] and NIH-3T3 cells expressing the rat ErbB3 cDNA [27] have been previously described.

Expression of EGF receptor and ErbB3 TKD forms

Recombinant human EGF receptor and rat ErbB3 TKD forms, each with a Met-His-His-His-His-His-His leader peptide, were expressed with the baculovirus/insect cell system. The hisTKD38-coding sequence was generated from the EGF receptor cDNA in pMMTV-ER [28] by PCR with the primers 5′-TGCTCTAGACCATGCACCACCACCACCACCACCGA-AGGCGCCACATCGTTCGG-3′ (forward) and 5′-CCCCCG-GGCTAGTTGGAGTCTGTAGGACTTGGCAA-3′ (reverse). The forward primer included an *Xba*I restriction site, a start codon (underlined) and six His codons, as well as the coding sequence for amino acid residues 645–651 of the EGF receptor. The reverse primer was complementary to the coding sequence for residues 965–972 of the EGF receptor, and introduced a stop codon (underlined) and a *Sma*I restriction site into the PCR product. The resulting PCR product was subcloned into the baculovirus transfer vector pAcYMP1 [29] to yield pAc-TKD38. A baculovirus transfer vector for hisTKD61 (pAc-TKD61) was generated by cloning a cDNA fragment encoding the EGF receptor C-terminus into pAc-TKD38.

The coding sequence for the rat ErbB3 TKD was amplified by PCR from a previously characterized rat ErbB3 cDNA clone, pBS-rB3 [27]. The forward primer, 5′-TGCTCTAGACCATG-CACCACCACCACCACCACCGAATTCGGATTCAGAA-CAAAAGGGCTA-3′, included an *Xba*I site, a start codon (underlined), six His codons and the codons for amino acid residues 668–674 of ErbB3. The reverse primer, 5′-ACAA-GCTGCAGAGATGAC-3′, was complementary to a coding sequence within the rat ErbB3 cDNA downstream of a unique *Nde*I restriction site. The resulting PCR product was cloned into pBS-rB3 to yield a cDNA encoding the hisTKD-B3 protein, which was then subcloned into pAcYMP1. The authenticity of the PCR-amplified sequences present in each transfer vector was directly verified by DNA sequencing.

The purified baculovirus transfer vectors were co-transfected with BaculoGold baculovirus DNA (Pharmingen) into cultured Sf21 cells [30]. Recombinant baculovirus clones were isolated by an end point dilution method [31], and viral clones expressing high levels of the recombinant TKDs were identified by immunoblotting lysates of virally infected Sf21 cells.

For large-scale preparation of recombinant proteins, Sf21 cells were grown in spinner flask culture (125 ml) to a density of (1–2) × 10^6 cells/ml, then infected with recombinant virus (∼ 10 plaque-forming units/cell) [30]. At 48 h after infection, cells were harvested and washed gently in 20 ml of insect cell lysis buffer (20 mM Tris/HCl, 0.5 M NaCl, 5 mM imidazole, 1 μg/ml pepstatin A, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM PMSF, pH 7.9) supplemented with 250 mM sucrose. The cells were resuspended in 10 ml of sucrose-free lysis buffer and sonicated. The homogenate was clarified by centrifugation for 20 min at 80000 ***g***, and supplemented with Triton X-100 to a final concentration of 0.05 %. The solution was applied to a 5 ml iminodiacetic acid–Sepharose 6B column (Sigma) that had been charged with 50 mM nickel sulphate and equilibrated with binding buffer (20 mM Tris/HCl, 0.5 M NaCl, 0.05 % Triton X-100, pH 7.9) supplemented with 5 mM imidazole. The column was washed with ten column volumes of binding buffer (5 mM imidazole) and six column volumes of binding buffer supplemented with 60 mM imidazole, then eluted with binding buffer supplemented with 250 mM imidazole. Peak fractions in the eluate were identified by protein assays [32] and pooled. Free imidazole was removed by extensive dialysis against TKD dialysis buffer [20 mM Tris/HCl, 100 mM NaCl, 10 % (v/v) glycerol, 0.05 % Triton X-100, pH 7.9]. The purified TKD forms (∼ 95 % pure, typically 1–2 mg of total protein) were supplemented with dithiothreitol to 1 mM and glycerol to 45 % (v/v) and stored at −20 °C. All purification steps were carried out at 4 °C or on ice. In protein purifications for CD measurements, Triton X-100 was omitted from the column elution and final dialysis buffers.

In vitro protein tyrosine kinase assays

TKD proteins (0.25 μM) were incubated for 5 min at room temperature in TKD dialysis buffer (36 μl total volume) with 15 μM [γ-^{32}P]ATP (∼ 10^4 c.p.m./pmol), 10 mM $MnCl_2$ or $MgCl_2$, and 0.1 % Triton X-100 added to the indicated concentrations. After quenching of the reactions by the addition of SDS/PAGE sample buffer, phosphoproteins were resolved by SDS/PAGE [33] and detected by autoradiography. Assays of exogenous peptide-phosphorylation activity included GST–TK7 (5 μg), a glutathione S-transferase fusion protein incorporating residues 943–1011 of the EGF receptor protein that has previously been shown to be an excellent protein tyrosine kinase substrate [26].

The cross-phosphorylation of the ErbB3 TKD (hisTKD-B3) by the truncated EGF receptor TKD (hisTKD38) was assayed as described above, except that the incubations were carried out for 15 min at room temperature. Phosphoproteins were then identified either by immunoblotting with the phosphotyrosine-specific antibody PY20 or by autoradiography. The kinetics of the cross-phosphorylation reaction were assayed by incubating hisTKD38 (0.25 μM) in the presence of 10 mM $MnCl_2$, 15 μM [γ-^{32}P]ATP and various concentrations (0–2.5 μM) of hisTKD-B3 for 5 min at room temperature in TKD dialysis buffer. The final glycerol concentrations of the samples were adjusted to a constant 30 % (v/v). The ^{32}P-labelled phosphoproteins were resolved by SDS/PAGE, identified by autoradiography, and quantified by scintillation counting of bands excised from dried gels. V_{max} and K_m were determined by the fitting of rate equations with a non-linear least-squares minimization alogrithm [34], and the hyperbolic curve generated is shown in Figure 6(B).

In immune complex kinase assays, immunoprecipitates were incubated with [γ-^{32}P]ATP (∼ 10^4 c.p.m./pmol) for 10 min at room temperature. Reactions contained 40 mM Hepes/Na (pH 7.4), 0.05 % Triton X-100, 10 mM $MnCl_2$, or 10 mM $MgCl_2$ and 3 mM $MnCl_2$, and 17 μM ATP. Then 5 × SDS/PAGE sample buffer was added to stop the reactions, and the samples were subjected to electrophoresis and autoradiography.

Fluorescence spectroscopic analysis of nucleotide binding

Binding of the TNP-ATP nucleotide analogue to the recombinant TKDs was analysed by a recently described fluorescence assay [25]. Briefly, fixed concentrations of recombinant protein were titrated with increasing concentrations of TNP–ATP (0–7.5 μM) as the fluorescence of the nucleotide was recorded. Fluorescence titration data were corrected for the contribution of both free and non-specifically bound TNP-ATP, as determined by titrations performed with the inclusion of excess ATP, and for inner filter quenching effects observed at high TNP-ATP concentrations. Dissociation constants for TNP-ATP binding were subsequently determined by fitting of a theoretical binding equation to the titration data [25].

CD spectroscopic measurements

UV CD spectra of recombinant proteins were recorded with an Aviv 62DS instrument with solutions of 2 μM protein in 10 mM Tris/HCl/50 mM NaCl/25 % (v/v) glycerol, pH 7.9, held in 2 mm cells thermostatically controlled at 4 °C. A solvent blank spectrum was subtracted from each protein spectrum. Analysis of CD spectra for determination of the content of secondary-structural elements was carried out with the aid of spectral decomposition software [35].

RESULTS

Generation and characterization of recombinant EGF receptor and ErbB3 TKDs

In order to compare the catalytic properties of the EGF receptor and the ErbB3 protein, the TKDs of these receptors were expressed as recombinant proteins with the baculovirus/insect cell system. Baculovirus expression vectors for two distinct EGF receptor TKD forms, one with an authentic C-terminus (hisTKD61) and one with a highly truncated C-terminus (hisTKD38), and a full-length ErbB3 TKD form (hisTKD-B3) were constructed (Figure 1). The three recombinant TKDs were expressed in Sf21 cells, and each of these proteins was effectively purified by Ni^{2+}-chelating column chromatography (Figure 2A).

The secondary structures of the purified recombinant proteins were analysed by CD spectroscopy. The spectra of the C-terminally complete hisTKD61 and hisTKD-B3 proteins were qualitatively similar, and spectral decomposition analysis [35] indicated similar contents of α-helix, β-sheet, β-turn and random structural elements (Figure 2B). Given that the EGF receptor-derived hisTKD61 protein was found to possess a catalytic activity comparable with that of the native EGF receptor protein (results not shown), it was assumed that this recombinant protein was folded in a native conformation. The similarity of the CD spectrum of the hisTKD-B3 protein to that of the hisTKD61 protein then suggested that the ErbB3-derived protein also assumed a native conformation.

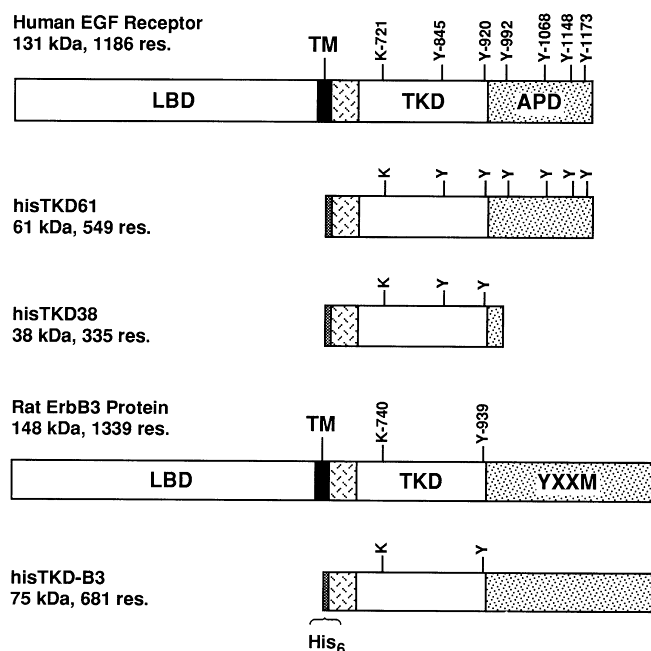

Figure 1 Schematic structures of recombinant EGF and ErbB3 receptor TKDs

The putative functional domains of the two receptor proteins are indicated: ligand-binding domain (LBD), transmembrane domain (TM), protein tyrosine kinase domain (TKD) and autophosphorylation domain (APD). Candidate phosphorylation sites in the EGF receptor are identified, and YXXM labels the C-terminal domain of ErbB3 that contains seven repetitions of the consensus phosphatidylinositol 3-kinase-binding site, Tyr-Xaa-Xaa-Met. The Met-His-His-His-His-His-His leader peptide introduced into each of the recombinant proteins is also indicated (His_6).

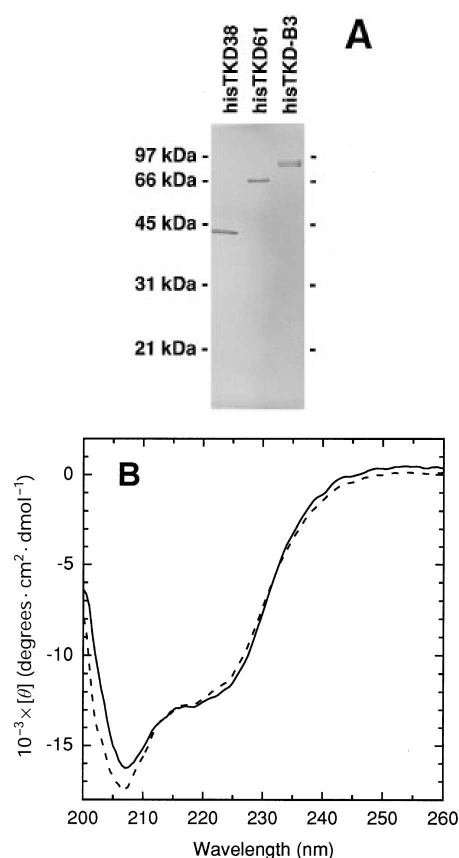

Figure 2 Characterization of EGF receptor and ErbB3 TKDs by SDS/PAGE and CD spectroscopy

(**A**) Recombinant TKD proteins generated with the baculovirus/insect cell system were purified to near-homogeneity by Ni^{2+}-chelating column chromatography (see the Experimental section). SDS/PAGE analysis with silver staining of 0.5 μg samples of the three TKD forms is shown. (**B**) CD spectra of EGF receptor (——) and ErbB3 (– – – –) TKDs were recorded and analysed for context of secondary-structural elements (see the Experimental section). Percentages of α-helix, β-sheet, β-turn and random elements were 41, 35, 10 and 14% respectively for the hisTKD61 protein, and 40, 38, 9 and 13% respectively for the hisTKD-B3 protein.

Catalytic activities of recombinant EGF receptor and ErbB3 TKDs

Previous studies of a full-length EGF receptor TKD expressed in the baculovirus/insect cell system indicated that the hisTKD61 protein would be an active protein tyrosine kinase showing selectivity for Mn^{2+} over Mg^{2+} as an activating metal ion [36,37]. The hisTKD38 protein was also expected to be fully active, although it was expected that this truncated protein would lack the strong autophosphorylation activity of the full-length TKD. The autophosphorylation and substrate phosphorylation activities of the two recombinant EGF receptor TKD forms were compared with those of the ErbB3-derived protein (Figure 3).

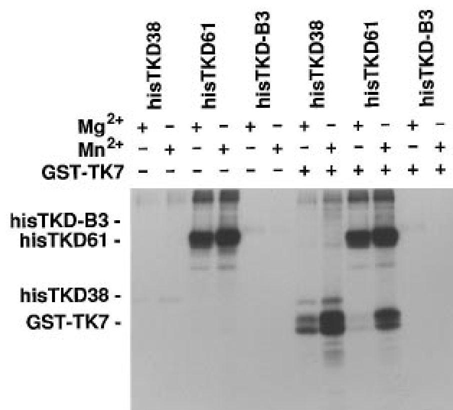

Figure 3 Autophosphorylation and substrate-phosphorylation activities of the EGF receptor and ErbB3 TKDs

Each of the TKD proteins (0.25 μM) was incubated for 5 min at room temperature in the presence of 15 μM [γ-^{32}P]ATP, and either 10 mM $MgCl_2$ or 10 mM $MnCl_2$ as indicated. TKD autophosphorylation was analysed by SDS/PAGE and autoradiography. Substrate phosphorylation activities of the TKD proteins were similarly assayed with the inclusion of 5 μg of the protein substrate GST–TK7 in the incubation as indicated. The GST–TK7 protein shows multiple bands when phosphorylated.

These experiments employed a recombinant fusion protein (GST–TK7) known to be a substrate for the EGF receptor and c-Src protein tyrosine kinases [26], and both Mg^{2+} and Mn^{2+} were tested as activators of the phosphorylation reactions.

Whereas the hisTKD61 protein showed a strong autophosphorylation, autophosphorylation of the hisTKD38 and hisTKD-B3 proteins was much weaker. Phosphoamino acid analyses (results not shown) indicated that, whereas the weak autophosphorylation of the hisTKD38 protein corresponded to the incorporation of phosphotyrosine, the hisTKD-B3 protein was not phosphorylated on tyrosine residues (see also Figure 6A). Each of the three TKD forms was phosphorylated to a very small extent on serine and threonine, which was apparently due to a contaminating serine/threonine kinase activity. Significantly, the substrate-phosphorylation activity of the hisTKD-B3 protein was negligible compared with that of the EGF receptor-derived TKDs. Several other attempts to detect protein tyrosine kinase activity in the ErbB3 TKD also yielded negative results. For example, when a distinct ErbB3 TKD lacking the hexa-His leader peptide was generated with a vaccinia virus expression system, intrinsic protein tyrosine kinase activity was again not evident (results not shown).

Catalytic activity of the full-length ErbB3 protein *in vitro*

The protein tyrosine kinase activity of the full-length ErbB3 protein was also assessed. Here, the native ErbB3 protein was immunoprecipitated from cells expressing the protein at a high level either as a consequence of gene transfection (3T3-B3 cells) or tumorigenesis (MDA-MB-453 and SK-BR-3) (Figure 4A). For comparison, the EGF receptor was immunoprecipitated from MDA-MB-468 cells. Immunoprecipitated proteins were incubated with [γ-^{32}P]ATP and bivalent metal ions. As expected, EGF receptor immune complexes showed strong autophosphorylation. In contrast, ErbB3 immunoprecipitates exhibited negligible autophosphorylation activity (Figure 4B). Neither varying the assay conditions nor stimulating with the ligand neuregulin led to the detection of ErbB3 kinase activity (results not shown). Both rat and human ErbB3 proteins were

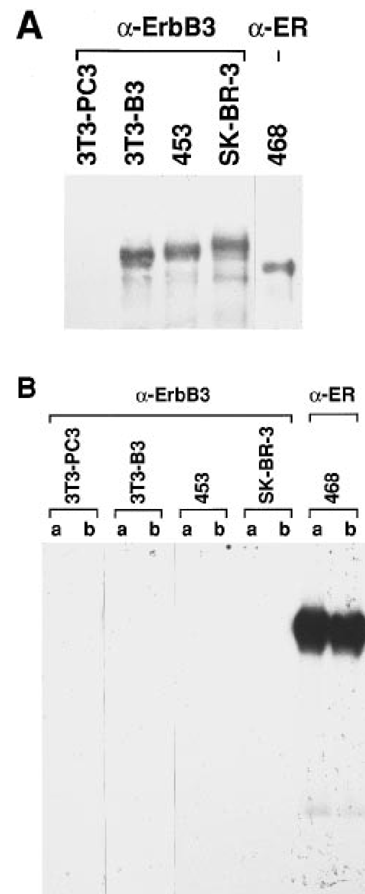

Figure 4 Autophosphorylation activities of full-length EGF receptor and ErbB3 proteins

(**A**) Immunoprecipitation of ErbB3 and EGF receptor from cultured human breast cancer cell lines and NIH-3T3 cells expressing recombinant rat ErbB3 protein. Detergent lysates were prepared from NIH-3T3 fibroblasts transfected with the parent pcDNA3 expression vector (3T3-PC3) or the pcDNA3-ErbB3 vector (3T3-B3) and three human breast cancer cell lines: MDA-MB-453 (453), MDA-MB-468 (468) and SK-BR-3. Aliquots of each lysate (4 mg of protein) were precleared with Protein G–agarose, then immunoprecipitated with either ErbB3-specific antibody 2F12 (α-ErbB3) or EGF-receptor-specific antibody LA1 (α-ER) as indicated. After two washes, precipitates were resuspended and one-half of each sample was analysed by SDS/PAGE and immunoblotting with the immunoprecipitating antibodies. (**B**) Immune complex kinase assays performed with ErbB3 and EGF receptor immunoprecipitates. One-fifth of each suspended immunoprecipitate analysed in (**A**) was incubated for 10 min at room temperature in the presence of 17 μM [γ-^{32}P]ATP and either 10 mM $MnCl_2$ (lanes a) or a mixture of 10 mM $MgCl_2$ and 3 mM $MnCl_2$ (lanes b). The phosphoproteins were resolved by SDS/PAGE and identified by autoradiography.

tested here, as the transfected NIH-3T3 cells expressed the rat ErbB3 protein and the cancer cell lines used were derived from human breast carcinomas.

Nucleotide-binding properties of EGF receptor and ErbB3 TKD proteins

Previously, we have shown that the fluorescent nucleotide analogue TNP–ATP binds to recombinant EGF receptor TKD forms, and that this binding can be conveniently monitored by measuring the enhancement of TNP-ATP fluorescence that occurs on binding to the TKD [25]. The Mn·TNP-ATP complex was found to be a functional substrate for the EGF receptor protein tyrosine kinase, which apparently mimics the authentic substrate Mn·ATP. The TNP-ATP nucleotide binding exhibited

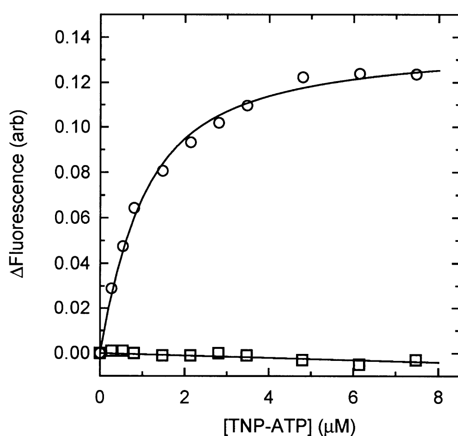

Figure 5 Nucleotide-binding properties of recombinant EGF receptor and ErbB3 TKDs

The interaction of the nucleotide analogue TNP-ATP with the recombinant TKD proteins was analysed by fluorescence spectroscopy as previously described [25]. Whereas the EGF-receptor-derived hisTKD61 protein (○) showed a high-affinity interaction with TNP-ATP ($K_d = 0.75 \pm 0.24$ μM), the ErbB3-derived hisTKD-B3 protein (□) showed no interaction.

by the EGF-receptor-derived hisTKD61 protein was directly compared with that of the hisTKD-B3 protein (see Figure 5). Whereas the hisTKD61 protein bound the nucleotide analogue with a dissociation constant in the micromolar range ($K_d = 0.75 \pm 0.24$ μM), there was no detectable interaction of the nucleotide analogue with the ErbB3-derived protein. Failure of the fluorescent nucleotide analogue to interact with hisTKD-B3 precluded attempts to address directly the ATP and Mn·ATP binding properties of this protein. However, the inability of the ErbB3 protein to bind the nucleotide analogue was certainly consistent with its observed lack of protein tyrosine kinase activity. In related studies (results not shown), a truncated ErbB3 TKD protein lacking the C-terminal phosphorylation domain was found to associate with TNP-ATP and ATP, but did not detectably interact with Mn·TNP-ATP and also showed no catalytic activity.

ErbB3 as a protein tyrosine kinase substrate

In our earlier work [38], C-terminal sequences of the ErbB3 receptor protein were found to be excellent substrates for the EGF receptor protein tyrosine kinase with K_m values ranging from 1 to 30 μM. This suggested that if the ErbB3 receptor was not itself an active protein kinase, it might serve as a substrate for another receptor protein kinase in the ErbB family. To examine the potential for EGF receptor/ErbB3 cross-phosphorylation, the EGF receptor-derived hisTKD38 protein was incubated with the ErbB3-derived hisTKD-B3 under phosphorylating conditions (Figure 6A). Whereas the hisTKD38 and hisTKD-B3 proteins alone showed negligible autophosphorylation activities when compared with the C-terminally complete hisTKD61 protein, hisTKD-B3 was strongly phosphorylated on incubation with hisTKD38. This phosphorylation could be detected by either autoradiography of ^{32}P-labelled proteins or immunoblotting with anti-phosphotyrosine (Figure 6A). The K_m and V_{max} for phosphorylation of the hisTKD-B3 substrate by the hisTKD38 protein kinase were approx. 0.5 μM and 1.4 nmol/min per mg respectively (Figure 6B). Hence the hisTKD-B3 protein exhibited a K_m value among the lowest documented for substrates for the EGF receptor protein tyrosine kinase. The hisTKD-B3

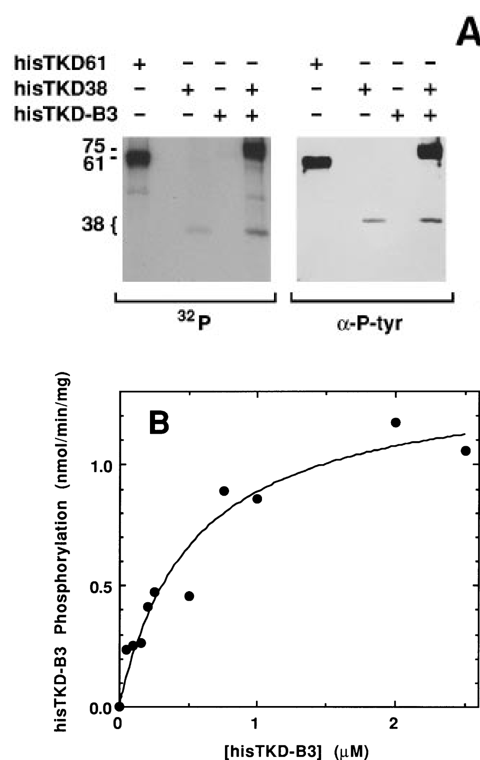

Figure 6 Autophosphorylation and cross-phosphorylation activities of EGF receptor and ErbB3 TKDs

(**A**) The C-terminally complete EGF receptor TKD (hisTKD61), the truncated EGF receptor TKD (hisTKD38) and the ErbB3 TKD (hisTKD-B3) (see Figure 1) (each at 0.25 μM concentration) were incubated either separately or together as indicated for 15 min at room temperature in the presence of 10 mM $MnCl_2$ and either 15 μM [γ-^{32}P]ATP (left panel) or 15 μM ATP (right panel). Phosphorylation was analysed by SDS/PAGE and autoradiography (left panel) or by anti-phosphotyrosine immunoblotting (right panel). (**B**) The kinetics of phosphorylation of hisTKD-B3 by hisTKD38 were analysed by incubating hisTKD38 (0.25 μM) in the presence of 0–2.5 μM hisTKD-B3 for 5 min at room temperature as in (**A**). ErbB3 phosphorylation was assayed by SDS/PAGE, autoradiography and scintillation counting of excised gel bands. The hyperbolic curve best fitting the experimental data is shown ($K_m = 0.54$ μM; $V_{max} = 1.4$ nmol/min per mg).

protein was also efficiently phosphorylated by the C-terminally complete EGF receptor protein kinase, hisTKD61 (results not shown), although the similar SDS/PAGE mobilities of the hisTKD-B3 and hisTKD61 proteins precluded a quantitative analysis of this phosphorylation reaction.

DISCUSSION

The *ErbB3* gene product has been predicted to be a receptor protein tyrosine kinase similar in structure and function to other EGF receptor family members [1,2]. We have attempted to detect intrinsic protein tyrosine kinase activity in the ErbB3 protein by various approaches. As the protein tyrosine kinase domains of a variety of other receptors have been produced in catalytically active form with the baculovirus system [29,39,40–42], we used this sytem in the generation of an ErbB3 cytosolic domain protein (hisTKD-B3). CD spectroscopic measurements indicated that the hisTKD-B3 protein was folded in a conformation similar to that of the corresponding EGF receptor cytosolic domain (hisTKD61), which displayed robust catalytic activity. However, the recombinant ErbB3 protein exhibited negligible catalytic activity under the same experimental conditions (Figure

3). Immune complex kinase assays of full-length ErbB3 proteins also failed to demonstrate intrinsic kinase activity (Figure 4). These results led to the conclusion that the ErbB3 protein is not intrinsically a protein kinase.

The potential of the ErbB3 protein to bind nucleotide substrates was assessed with the aid of the fluorescent nucleotide analogue TNP-ATP, which has previously been used to characterize the nucleotide-binding properties of the EGF receptor TKD [25]. Whereas the EGF receptor-derived hisTKD61 protein again bound TNP-ATP with high affinity (Figure 5), there was no observed enhancement of TNP-ATP fluorescence in the presence of the hisTKD-B3 protein. Because there was no apparent interaction of the analgoue with hisTKD-B3, it was not possible to use this assay to investigate the potential interaction of ErbB3 with the authentic substrate Mn·ATP. In a previous study of recombinant bovine ErbB3 [7], the receptor protein was seen to be specifically labelled by 5′-*p*-fluorosulphonylbenzoyladenosine, although again no evidence for intrinsic kinase activity was obtained. Given that the ErbB3 cytosolic domain here did not interact with the analogue TNP-ATP and also showed no catalytic activity, it is reasonable to suspect that the ErbB3 protein may be unable to bind ATP in the same manner as other protein tyrosine kinases.

The apparent absence of catalytic activity and failure to bind nucleotide substrate might be explained by the occurrence of non-conservative amino acid substitutions in the putative protein tyrosine kinase domain of ErbB3. Specifically, the residues Cys-721, His-740 and Asn-815 in human ErbB3 [2] correspond to Ala, Glu and Asp respectively in all other known protein tyrosine kinases [4]. Sequencing of the rat ErbB3 cDNA has revealed an Asp residue corresponding to Asn-815 in human ErbB3 [27], which suggested that the rat ErbB3 protein, unlike human ErbB3, might possess kinase activity. However, neither rat nor human ErbB3 showed evidence of catalytic activity in this study (Figure 4).

A previous study of the bovine ErbB3 protein also yielded no indication of significant kinase activity [7]. However, an apparent intrinsic protein tyrosine kinase activity was detected in other investigations of the human ErbB3 protein [5,6]. In these latter studies, *in vitro* phosphorylation of ErbB3 in an immune complex [5] and EGF-stimulated *in vivo* phosphorylation of a chimaeric receptor consisting of the extracellular domain of EGF receptor and cytosolic domain of ErbB3 were demonstrated [5,6]. It is possible that this observed ErbB3 phosphorylation resulted from the action of an associated non-ErbB3 kinase. For example, the *in vivo* and *in vitro* phosphorylations of kinase-deficient mutant forms of the EGF receptor have been demonstrated [43,44], and an ectopically expressed kinase-deficient EGF receptor mutant was shown to be cross-phosphorylated by endogenous wild-type EGF receptors [45].

The ErbB3 protein has been shown to function with ErbB2/neu as a high-affinity coreceptor for the neuregulin (heregulin) peptides [8,10]. Also, the EGF-dependent phosphorylation of the ErbB3 protein in human cancer cells expressing high levels of both EGF receptor and ErbB3 has been documented [11,12]. A variety of recent evidence is consistent with a general model in which pairs of distinct ErbB family receptor proteins function as receptor heterodimers [23]. In this model, receptor heterodimerization provides a mechanism for diversifying the signal-transduction pathways activated by polypeptide growth factors in the EGF family. As specific phosphorylated tyrosine residues within the unique C-termini of the ErbB family members have been shown to function as docking sites for distinct signal-transducing proteins such as phospholipase C, phosphatidylinositol 3-kinase, Grb2 and Shc [46], receptor phosphorylation in the context of heterodimers is a critical event in ErbB family receptor signal transduction. If devoid of intrinsic protein tyrosine kinase activity, the ErBB3 protein would be phosphorylated only in association with other ErbB family receptor proteins. Our observation that the ErbB3 protein was an excellent substrate for the EGF receptor *in vitro* (Figure 6) is consistent with the assumption that the documented *in vivo* phosphorylation of ErbB3 in response to either EGF or neuregulin results directly from the action of other ErbB family protein tyrosine kinases.

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REFERENCES

1 Kraus, M. H., Issing, W., Miki, T., Popescu, N. C. and Aaronson, S. A. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 9193–9197
2 Plowman, G. D., Whitney, G. S., Neubauer, M. G., Green, J. M., McDonald, V. L., Todaro, G. J. and Shoyab, M. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 4905–4909
3 Fantl, W. J., Johnson, D. E. and Williams, L. T. (1993) Annu. Rev. Biochem. **62**, 453–481
4 Hanks, S. K. and Quinn, A. M. (1991) Methods Enzymol. **200**, 38–62
5 Kraus, M. H., Fedi, P., Starks, V., Muraro, R. and Aaronson, S. A. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 2900–2904
6 Prigent, S. A. and Gullick, W. J. (1994) EMBO J. **13**, 2831–2841
7 Guy, P. M., Platko, J. V., Cantley, L. C., Cerione, R. A. and Carraway, III, K. L. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 8132–8136
8 Carraway, III, K. L., Sliwkowski, M. X., Akita, R., Platko, J. V., Guy, P. M., Nuijens, A., Diamonti, A. J., Vandlen, R. L., Cantley, L. C. and Cerione, R. A. (1994) J. Biol. Chem. **269**, 14303–14306
9 Sliwkowski, M. X., Schaefer, G., Akita, R. W., Lofgren, J. A., Fitzpatrick, V. D., Nuijens, A., Fendly, B. M., Cerione, R. A., Vandlen, R. L. and Carraway, III, K. L. (1994) J. Biol. Chem. **269**, 14661–14665
10 Tzahar, E., Levkowitz, G., Karaunagaran, D., Yi, L., Peles, E., Lavi, S., Chang, D., Liu, N., Yayon, A., Wen, D. and Yarden, Y. (1994) J. Biol. Chem. **269**, 25226–25233
11 Soltoff, S. P., Carraway, III, K. L., Prigent, S. A., Gullick, W. G. and Cantley, L. C. (1994) Mol. Cell. Biol. **14**, 3550–3558
12 Kim, H.-H., Sierke, S. L. and Koland, J. G. (1994) J. Biol. Chem. **269**, 24747–24755
13 Kita, Y. A., Barff, J., Luo, Y., Wen, D., Brankow, D., Hu, S., Liu, N., Prigent, S. A., Gullick, W. J. and Nicolson, M. (1994) FEBS Lett. **349**, 139–143
14 Peles, E., Ben-Levy, R., Tzahar, E., Liu, N., Wen, D. and Yarden, Y. (1993) EMBO J. **12**, 961–971
15 Wallasch, C., Weiss, F. U., Niederfellner, G., Jallal, B., Issing, W. and Ullrich, A. (1995) EMBO J. **14**, 4267–4275
16 Riese, II, D. J., van Raaij, T. M., Plowman, G. D., Andrews, G. C. and Stern, D. F. (1995) Mol. Cell. Biol. **15**, 5770–5776
17 Beerli, R. R., Graus-Porta, D., Woods-Cook, K., Chen, X. M., Yarden, Y. and Hynes, N. E. (1995) Mol. Cell. Biol. **15**, 6496–6505
18 Zhang, K., Sun, J. L., Liu, N. L., Wen, D. Z., Chang, D., Thomason, A. and Yoshinaga, S. K. (1996) J. Biol. Chem. **271**, 3884–3890
19 Pinkas-Kramarski, R., Soussan, L., Waterman, H., Levkowitz, G., Alroy, I., Klapper, L., Lavi, S., Seger, R., Ratzkin, B. J., Sela, M. and Yarden, Y. (1996) EMBO J. **15**, 2452–2467
20 Pinkas-Kramarski, R., Shelly, M., Glathe, S., Ratzkin, B. J. and Yarden, Y. (1996) J. Biol. Chem. **271**, 19029–19032
21 Tzahar, E., Waterman, H., Chen, X., Levkowitz, G., Karunagaran, D., Lavi, S., Ratzkin, B. J. and Yarden, Y. (1996) Mol. Cell. Biol. **16**, 5276–5287
22 Carraway, III, K. L. and Cantley, L. C. (1994) Cell **78**, 5–8
23 Lemmon, M. A. and Schlessinger, J. (1994) Trends Biochem. Sci. **19**, 459–463
24 Hynes, N. E. and Stern, D. F. (1994) Biochim. Biophys. Acta Rev. Cancer **1198**, 165–184
25 Cheng, K. R. and Koland, J. G. (1996) J. Biol. Chem. **271**, 311–318
26 Koland, J. G., O'Brien, K. M. and Cerione, R. A. (1990) Biochem. Biophys. Res. Commun. **166**, 90–100
27 Hellyer, N. J., Kim, H.-H., Graves, C. H., Sierke, S. L. and Koland, J. G. (1995) Gene **165**, 279–284
28 Clark, A. J. L., Beguinot, L., Ishii, S., Ma, D. P., Roe, B. A., Merlino, G. T. and Pastan, I. (1986) Biochim. Biophys. Acta **867**, 244–251
29 Guy, P. M., Carraway, III, K. L. and Cerione, R. A. (1992) J. Biol. Chem. **267**, 13851–13856

30 Summers, M. D. and Smith, G. E. (1987) A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures, Texas Agriculture Experimental Station, College Station, Texas
31 Fung, M.-C., Chiu, K. Y. M., Weber, T., Chang, T.-W. and Chang, N. T. (1988) J. Virol. Methods **19**, 33–42
32 Bradford, M. (1976) Anal. Biochem. **72**, 248–254
33 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
34 Nelder, J. A. and Mead, R. (1965) Comput. J. **7**, 308–313
35 Sreerama, N. and Woody, R. W. (1993) Anal. Biochem. **209**, 32–44
36 Wedegaertner, P. B. and Gill, G. N. (1989) J. Biol. Chem. **264**, 11346–11353
37 Koland, J. G. and Cerione, R. A. (1990) Biochim. Biophys. Acta **1052**, 489–498
38 Sierke, S. L. and Koland, J. G. (1993) Biochemistry **32**, 10102–10108
39 Cobb, M. H., Sang, B.-C., Gonzales, R., Goldsmith, E. and Ellis, L. (1989) J. Biol. Chem. **264**, 18701–18706
40 Herrera, R., Lebwohl, D., de Herreros, A. G., Kallen, R. G. and Rosen, O. M. (1988) J. Biol. Chem. **263**, 5560–5568
41 Wedegaertner, P. B. and Gill, G. N. (1992) Arch. Biochem. Biophys. **292**, 273–280
42 Wei, L., Hubbard, S. R., Hendrickson, W. A. and Ellis, L. (1995) J. Biol. Chem. **270**, 8122–8130
43 Selva, E., Raden, D. L. and Davis, R. J. (1993) J. Biol. Chem. **268**, 2250–2254
44 Coker, K. J., Staros, J. V. and Guyer, C. A. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 6967–6971
45 Hack, N., Sue-A-Quan, A., Mills, G. B. and Skorecki, K. L. (1993) J. Biol. Chem. **268**, 26441–26446
46 Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Leichleider, R. J., Neel, B. G., Birge, R. B., Fajardo, J. E., Chou, M. M., Hanafusa, H., Schaffhausen, B. and Cantley, L. C. (1993) Cell **72**, 767–778

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