Nucleotide-binding properties of kinase-deficient epidermal-growth-factor-receptor mutants

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The nucleotide-binding properties of wild-type epidermal-growth-factor (EGF)-receptor protein tyrosine kinase (PTK) and EGF-receptor mutants with site-specific amino acid substitutions known to attenuate protein kinase activity were analysed by a fluorescence competition assay employing the nucleotide analogue 2'(3')-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate. Binding affinities for ATP and Mn·ATP complex were determined for the PTK domains of the wild-type and two mutant proteins. Surprisingly, mutation of the highly conserved Lys-721 residue in the nucleotide-binding site of the EGF-

receptor PTK domain did not abolish ATP and Mn·ATP binding, although the binding affinity for the Mn·ATP complex was significantly reduced. A second kinase-inactivating mutation that targeted the highly conserved Asp-813 residue had little effect on the nucleotide-binding properties of the EGF-receptor PTK domain. These results indicated that the principle effect of these two kinase-inactivating amino acid substitutions is not to block nucleotide binding, but is instead an inhibition of the phospho-transfer reaction.

INTRODUCTION

A large number of proteins with intrinsic protein tyrosine kinase (PTK) activity have now been identified. These proteins include numerous polypeptide growth factor receptors that function in the normal control of cell growth and development and also participate in the abnormal growth of cancer cells [1,2]. Site-directed mutagenesis has been employed to demonstrate that intrinsic PTK activity is essential to the functions of these receptors (cf. [3–7]; but see also [8–12]). The substitution of certain highly conserved amino acid residues in the PTK catalytic domain, such as Lys-721 of the epidermal growth factor (EGF) receptor [6], has been shown to abolish or greatly attenuate catalytic activity. The three-dimensional X-ray structure of cyclic AMP-dependent protein kinase crystallized with bound ATP indicates that the corresponding Lys residue in this serine/threonine protein kinase interacts with the α - and β -phosphate oxygens of ATP [13]. As this Lys residue is conserved in all protein kinases with known amino acid sequence [14], it has been assumed to perform a similar function in the active sites of PTKs. Indeed, Lys-721 of the EGF receptor PTK has been shown to be specifically labelled with a reactive ATP analogue [15].

Substitution of Asp-813 of the conserved His-Arg-Asp motif of the catalytic domains of the EGF receptor [11] and v-Fps [16] PTKs has also been used in the ablation of PTK activity. It has been proposed that the homologous residue in the cyclic AMP-dependent protein kinase participates directly as a catalytic base in the phospho-transfer reaction [13]. Mutations of the corresponding Asp residue in the stem cell factor receptor PTK (product of the *c-kit* proto-oncogene) have been characterized, and in this context an Asp $\rightarrow$ Asn mutation inactivates PTK activity and disrupts signal transduction [17,18]. The human

ErbB3 protein, a receptor for the neuregulins and the third identified member of the EGF receptor family, presents a unique example of a putative PTK with naturally occurring amino acid substitutions for residues that are otherwise highly conserved. The residues Cys-721, His-740 and Asn-815 in ErbB3 correspond to Ala, Glu and Asp, respectively, in all other known PTKs, with this latter Asp residue an element of the His-Arg-Asp motif characteristic of known PTKs. It has been considered that these amino acid substitutions might explain why the ErbB3 protein has been seen to have an impaired catalytic activity relative to that of other EGF receptor family members [19,20].

Signal transduction events mediated by receptors with PTK activity include the activation of the mitogen-activated protein kinase (MAPK) cascade [21] and the stimulation of nuclear DNA synthesis. Recent studies of the signal-transducing potentials of kinase-deficient EGF receptors with mutations of either Lys-721 or Asp-813 have yielded surprising results. Whereas EGF receptors incorporating Lys-721 $\rightarrow$ Met [8] or Lys-721 $\rightarrow$ Arg [12] substitutions were both shown to activate the MAPK signalling pathway, these receptors did not stimulate mitogenesis. In contrast, an EGF receptor mutant with an Asp-813 $\rightarrow$ Ala substitution was found to stimulate DNA synthesis, as well as MAPK activity [11]. Hence, an EGF receptor mutant that was devoid of kinase activity could still activate mitogenic signal transduction pathways. It has been proposed that the marked differences in the signalling potentials of these two kinase-deficient receptor mutants might reflect differences in their nucleotide binding properties [11].

In order to understand better the effects that specific amino acid substitutions have on the catalytic and signal-transducing properties of PTKs, the nucleotide binding characteristics of recombinant EGF receptor PTK domains were examined by the

Abbreviations used: TNP-ATP, 2'(3')-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate; EGF, epidermal growth factor; PTK, protein tyrosine kinase; TKD61, recombinant EGF receptor cytosolic domain; TKD61-K/M, TKD61 protein with a Lys-721 $\rightarrow$ Met amino acid substitution; TKD61-D/A, TKD61 protein with a Asp-813 $\rightarrow$ Ala amino acid substitution; GAT, a random co-polymer of glutamate, alanine and tyrosine [(Glu:Ala:Tyr)_{6:3:1}]; MAPK, mitogen-activated protein kinase; FGF, fibroblast growth factor.

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application of a recently described fluorescence assay [22]. Both the wild-type protein and proteins incorporating either a Lys-721 → Met or Asp-813 → Ala mutation were studied. These two previously described kinase-inactivating mutations were found not to block the binding of nucleotide. It appeared that the Lys-721 → Met substitution instead affected catalytic activity by an alteration of active site structure. This structural perturbation was also evidenced by an attenuation of the intrinsic tryptophan fluorescence of the protein. The nucleotide binding and structural characteristics of the Asp-813 → Ala mutant protein were not appreciably altered, which indicated that this kinase-inactivating mutation effected a subtler defect in the catalysis of the phosphotransfer reaction.

EXPERIMENTAL

Materials

2'(3')-O-(2,4,6-Trinitrophenyl)adenosine 5'-triphosphate (TNP-ATP) was obtained from Molecular Probes, Inc. ATP sodium salt and (Glu:Ala:Tyr)_{6:3:1} (GAT), a random co-polymer of glutamate, alanine and tyrosine in the indicated molar ratio and with average M_r 43000, were purchased from Sigma Chemical Company. [γ -³²P]ATP (~3000 Ci/mmol) was supplied by Dupont–New England Nuclear.

Generation of recombinant receptor PTK domains

The generation and purification of recombinant human EGF receptor PTKs has been described in detail [20]. Briefly, the cytosolic domain of the EGF receptor PTK with a Met-His-His-His-His leader peptide was generated with the baculovirus/insect cell system and purified by metal-chelating chromatography. The wild-type TKD61 protein, incorporating amino acid residues 645–1186 of the human EGF receptor, included the predicted PTK domain and the C-terminal autophosphorylation domain. Full-length EGF receptor cDNAs containing mutations corresponding to the Lys-721 → Met [6] and Asp-813 → Ala [11] substitutions have been previously described, and baculovirus vectors encoding the cytosolic domains of these proteins (TKD61-K/M and TKD61-D/A) were generated by subcloning PCR-amplified fragments of the mutant EGF receptor cDNAs into baculovirus transfer vectors. The isolated recombinant proteins (~95% pure) were exhaustively dialysed against 20 mM Tris/HCl/100 mM NaCl/10% (v/v) glycerol/0.05% Triton X-100/pH 7.9, then supplemented with glycerol to 45% (v/v), and stored at –20 °C prior to use.

Catalytic activities of the recombinant PTK domains

The autophosphorylation activity of each recombinant PTK domain was assayed as follows. The PTK (10 pmol) was incubated in 20 mM sodium Hepes/10 mM MnCl₂/70 μ M [γ -³²P]ATP (~10⁴ c.p.m./pmol)/0.1% Triton X-100, pH 7.4, for 60 s at room temperature (final volume 36 μ l). The reaction was quenched by the addition of electrophoresis sample buffer and then subjected to SDS/PAGE. Incorporation of [³²P]P_i into the PTK was detected by autoradiography of dried gels. Substrate

phosphorylation activity assays employed the synthetic substrate, GAT [23]. PTKs (1.6 pmol TKD61, 16 pmol TKD61-K/M, or 16 pmol TKD61-D/A) were incubated in 20 mM sodium Hepes/10 mM MnCl₂/0.1 g/l BSA/0.1 g/l GAT/10% (v/v) glycerol, pH 7.4. Assays were initiated by the addition of [γ -³²P]ATP (~10⁴ c.p.m./pmol) to a concentration of 50 μ M. Following a 15-min incubation at room temperature, reactions were quenched by the addition of 10% trichloroacetic acid, and the incorporation of [³²P]P_i into GAT was quantified by a filter binding assay [23]. Activities are presented as the means for triplicate assays in units of nmol of phosphate incorporated into GAT/min per mg of TKD61 protein.

Fluorescence spectroscopic analysis of nucleotide binding

Binding of the TNP-ATP nucleotide analogue to the recombinant PTKs was analysed by a recently described fluorescence assay [22]. Briefly, fixed concentrations of recombinant PTK were titrated with increasing concentrations of TNP-ATP as the fluorescence of the nucleotide was recorded. Fluorescence titration data were corrected for the contribution of both free and nonspecifically bound TNP-ATP, as determined by titrations performed with the inclusion of excess ATP (10 mM), and for inner filter quenching effects observed at high TNP-ATP concentrations. ATP binding was monitored by TNP-ATP competition assays in which samples containing fixed concentrations of PTK and TNP-ATP were titrated with increasing concentrations of ATP. Dissociation constants for TNP-ATP and ATP binding were subsequently determined by fitting of a theoretical binding equation to titration data [22]. The binding characteristics of Mn·nucleotide complexes were determined by titrations performed in the presence of a fixed concentration (100 μ M) of Mn²⁺, with the concentrations of free and nucleotide-bound Mn²⁺ adjusted as previously described [24]. Here, non-specific binding was determined by titrations performed with 1 mM Mn·ATP in the medium.

CD and protein tryptophan fluorescence measurements

Near-UV CD spectra of recombinant PTKs were recorded with an Aviv 62DS instrument with solutions of 2 μ M protein in 10 mM Tris/HCl/50 mM NaCl/45% (v/v) glycerol, pH 7.9, held in 2-mm cells thermostated at 4 °C. A solvent blank spectrum was subtracted from each protein spectrum. Analysis of CD spectra for determination of the content of α -helical, β -sheet, turns and unordered structural elements was done with the aid of the spectral decomposition software of Sreerama and Woody [25]. Protein tryptophan fluorescence emission spectra were recorded with an SLM4800C instrument operating in ratio mode with excitation wavelength 280 nm and samples of 0.5 μ M protein in 20 mM sodium Hepes/10% (v/v) glycerol, pH 7.4, held in standard 1-cm cells.

RESULTS

Generation of EGF receptor PTK domains

In order to compare the nucleotide binding properties of the wild-type EGF receptor PTK with those of mutant EGF receptor proteins incorporating site-specific amino acid substitutions in the catalytic domain, the PTK domain of each of the proteins

Figure 1 Expression and purification of recombinant EGF receptor cytosolic domain proteins

The cytosolic domains of wild-type (TKD61) and mutant EGF receptor cytosolic domain proteins incorporating Lys-721 → Met (TKD61-K/M) and Asp-813 → Ala (TKD61-D/A) amino acid substitutions were expressed in the baculovirus/insect cell system and purified as previously described [20]. An SDS/PAGE analysis with Coomassie Blue staining of 4 μ g samples of each recombinant protein is shown.

was generated in the baculovirus/insect cell expression system [26]. These recombinant proteins, designated TKD61, TKD61-K/M and TKD61-D/A, contained the entire cytosolic domain of the EGF receptor (amino acid residues 645–1186). The TKD61-K/M and TKD61-D/A proteins incorporated Lys → Met and Asp → Ala substitutions at the residues corresponding to Lys-721 and Asp-813, respectively, in the full-length receptor protein. As shown in Figure 1, each of these recombinant proteins was effectively purified by Ni^{2+} -chelating column chromatography.

The structural integrity of the recombinant PTK domains was assessed by CD spectroscopy. The CD spectra of the three EGF receptor proteins were nearly identical, which indicated that the amino acid substitutions induced no gross perturbations of protein secondary structure (Figure 2A). Spectral analysis showed some differences in the fractional content of α -helical, β -sheet, turns and unordered structural elements between the proteins (Figure 2A, legend). However, as the estimates of these fractions would be sensitive to small errors in the determined protein concentrations and the possible presence of small quantities of denatured protein in the preparations, these differences were not considered to be significant. The intrinsic tryptophan fluorescence emission spectrum of each of the recombinant proteins was also recorded (Figure 2B). Whereas the wild-type TKD61 protein and the mutant TKD61-D/A protein showed similar tryptophan fluorescence spectra, the fluorescence of the TKD61-K/M mutant protein was significantly attenuated. It could be hypothesized that the substitution of Met for Lys-721 either significantly perturbed the folding of the polypeptide, or that the Lys-721 residue was in close proximity to a single Trp residue that contributed significantly to the overall the fluorescence emission of the PTK domain.

Figure 2 CD and intrinsic tryptophan fluorescence spectra of wild-type and mutant EGF receptor cytosolic domain proteins

(A) Near-UV CD spectra of the wild-type TKD61 (—), TKD61-K/M (---) and TKD61-D/A (· · ·) proteins were recorded and analysed as described in the Experimental section. Spectral decomposition analysis yielded the apparent percentages of α -helical (α), β -sheet (β_s), turns (t) and unordered (u) structural elements for TKD61 ($\alpha = 41\%$, $\beta_s = 35\%$, $t = 10\%$, $u = 14\%$), TKD61-K/M ($\alpha = 28\%$, $\beta_s = 35\%$, $t = 13\%$, $u = 22\%$), and TKD61-D/A ($\alpha = 26\%$, $\beta_s = 38\%$, $t = 12\%$, $u = 23\%$). (B) Intrinsic tryptophan emission spectra of the wild-type TKD61 (—), TKD61-K/M (---) and TKD61-D/A (· · ·) proteins are shown along with a solvent blank spectrum (— · —).

Catalytic properties of the recombinant PTKs

The PTK activity of the wild-type EGF receptor PTK domain (TKD61) has been previously characterized [20]. The autophosphorylation activity of this wild-type protein was compared with that of the mutant EGF receptor proteins (Figure 3). Whereas the wild-type EGF receptor TKD61 protein exhibited strong autophosphorylation activity, the TKD61-K/M and TKD61-D/A proteins showed negligible activity. Similar

Figure 3 Autophosphorylation activities of wild-type and mutant EGF receptor cytosolic domain proteins

TKD61, TKD61-K/M or TKD61-D/A (10 pmol) was incubated in the presence of 10 mM MnCl_2 and 70 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ for 1 min at room temperature, and autophosphorylation of the recombinant protein analysed by SDS/PAGE and autoradiography.

results were obtained when autophosphorylation was detected by immunoblotting with antiphosphotyrosine (data not shown). The substrate phosphorylation activities of the recombinant proteins were also assayed with the use of a polymeric tyrosine-containing peptide substrate. Here, the PTK activities of the Lys-721 $\rightarrow$ Met ($0.21 \pm 0.03 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$) and Asp-813 $\rightarrow$ Ala ($0.16 \pm 0.05 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$) mutant EGF receptor proteins were indistinguishable from the assay blank ($0.21 \pm 0.09 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$), which indicated that these proteins possessed activities less than 2% of the wild-type EGF receptor protein ($12.7 \pm 0.5 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$). Together these assay results confirmed earlier observations that the Lys-721 $\rightarrow$ Met and Asp-813 $\rightarrow$ Ala substitutions both render the EGF receptor PTK inactive.

Nucleotide-binding properties of the recombinant PTKs

We have previously described a convenient spectroscopic assay for nucleotide binding to the EGF receptor PTK, which employs the nucleotide analogue TNP-ATP [22]. In this assay, the enhancement of TNP-ATP fluorescence that occurs upon its binding to the PTK active site is monitored. Binding of the authentic substrate ATP is observed indirectly via its competition with TNP-ATP. Figure 4(A) shows the results of titrations in which the enhancement of TNP-ATP fluorescence was recorded as its concentration was varied in the presence of a fixed concentration of either wild-type or mutant PTK. In each case, a concentration-dependent enhancement of TNP-ATP fluorescence was observed that was indicative of TNP-ATP binding. The nonspecific component of TNP-ATP binding was determined by titrations performed in the presence of excess ATP, and the dissociation constants (K_d) characterizing the specific component of TNP-ATP binding to each of the proteins were determined by analysis of the titration curves (see the Experimental section). TNP-ATP binding was also evaluated by titrations performed with a fixed concentration (100 μM) of free bivalent metal ion, Mn^{2+} , present in the assay medium (Figure 4B).

Figure 4 Binding of TNP-ATP and $\text{Mn} \cdot \text{TNP-ATP}$ complex to wild-type and mutant EGF receptor cytosolic domain proteins

Solutions of either wild-type TKD61 ($\circ$), TKD61-K/M (∇) or TKD61-D/A ($\square$) [0.5 μM in (A) and 1.0 μM in (B)] were titrated with increasing concentrations of TNP-ATP as the fluorescence of the nucleotide analogue was recorded. Subtraction of data from similar titrations in which excess ATP was included yielded the specific enhancement of TNP-ATP fluorescence as is shown. The continuous lines are theoretical titration curves resulting from curve-fitting analysis, which also yielded the estimates for the dissociation constants given in Table 1. Titrations were performed without (A) or with (B) the inclusion of 100 μM free MnCl_2 in the assay medium.

Each of the recombinant EGF receptor PTK domains bound TNP-ATP with high affinity (Table 1). As previously observed with the wild-type EGF receptor PTK domain, the binding affinities exhibited in experiments with free Mn^{2+} present were significantly lower. It was presumed that the K_d values determined from these titrations reflected the binding affinities of the $\text{Mn} \cdot \text{TNP-ATP}$ complex. Dissociation constants for binding of ATP and $\text{Mn} \cdot \text{ATP}$ nucleotide complex to the recombinant

Table 1 Dissociation constants characterizing the binding of TNP-ATP and ATP to recombinant EGF receptor PTK domains

Dissociation constants for binding of TNP-ATP and ATP to wild-type (TKD61) and mutant EGF receptor PTK domains were estimated by fluorescence titrations (Figures 4 and 5) performed in the presence or absence of 100 μM free Mn^{2+} as previously described [22]. Data from at least two experiments were analysed in composite by curve fitting to yield the best-fit K_d values and S.E.M. estimates given. $K_{\text{MnTNP-ATP}}$ values are given as averages $\pm$ S.E.M. of at least six independent determinations. All values are given as μM .

	$K_{\text{TNP-ATP}}$	$K_{\text{MnTNP-ATP}}$	K_{ATP}	K_{MnATP}
TKD61	0.75 ± 0.24	12.3 ± 3.0	660 ± 63	6.1 ± 0.8
TKD61-D/A	0.35 ± 0.12	4.4 ± 1.2	309 ± 25	3.7 ± 0.7
TKD61-K/M	0.45 ± 0.22	8.2 ± 1.7	334 ± 27	100 ± 23

PTKs were determined by competition titrations in which the fluorescence of bound TNP-ATP was monitored as increasing concentrations of ATP or $\text{Mn} \cdot \text{ATP}$ were added (Figure 5). In the cases of the wild-type PTK, TKD61, and the TKD61-D/A mutant, the $\text{Mn} \cdot \text{ATP}$ complex was bound more tightly than the free nucleotide (see Table 1). The K_d values for binding of $\text{Mn} \cdot \text{ATP}$ to the wild-type and Asp-813 $\rightarrow$ Ala mutant proteins were similar to the K_m for the substrate complex in the auto-phosphorylation reaction of the PTK ($K_m \sim 6 \mu\text{M}$ [22]). In contrast, the TKD61-K/M mutant protein showed a greatly reduced affinity for the $\text{Mn} \cdot \text{ATP}$ complex.

DISCUSSION

Protein tyrosine kinase (PTK) activity is intrinsic to the receptors for a diverse group of polypeptide growth factors and developmental factors. Site-directed mutagenesis has been used to ablate the PTK activities of a variety of these receptors, in order to ascertain the role of this activity in receptor signalling. Naturally occurring mutations that attenuate PTK activity have also been observed. In general, the loss of PTK activity appears to result in the disruption of normal signal transduction. However, some examples of kinase-deficient mutant proteins that still activate cellular signalling pathways have been described. EGF receptor mutants with either Lys-721 $\rightarrow$ Met or Asp-813 $\rightarrow$ Ala substitutions appear to be devoid of intrinsic kinase activity, yet are still capable of mediating an EGF-dependent activation of the MAPK signalling pathway [8,11]. Whereas the Lys-721 $\rightarrow$ Met mutant receptor protein could not relay a full mitogenic signal [8], an EGF-dependent stimulation of DNA synthesis was mediated by the Asp-813 $\rightarrow$ Ala mutant receptor [11].

In the present work, we have characterized the nucleotide binding properties of EGF receptor proteins by application of a spectroscopic assay that employs the fluorescent nucleotide analogue TNP-ATP. Recombinant proteins corresponding to the cytosolic domain of the receptor and incorporating the predicted PTK catalytic domain were generated with the baculovirus/insect cell system. The results of these experiments indicated that mutant EGF receptor proteins incorporating either the Lys-721 $\rightarrow$ Met (TKD61-K/M) or Asp-813 $\rightarrow$ Ala (TKD61-D/A) amino acid substitution were still capable of binding ATP with near normal affinity (Table 1). Both mutant proteins were also able to bind a bivalent metal ion–nucleotide complex, which is presumed to be the active form of the nucleotide substrate. However, it was observed that the dissociation constant for binding of the $\text{Mn} \cdot \text{ATP}$ complex to the Lys-721 $\rightarrow$ Met mutant protein (100 μM) was significantly greater than that for the wild-type (6.1 μM) and Asp-813 $\rightarrow$ Ala mutant (3.7 μM) proteins. Hence,

Figure 5 Binding of ATP and $\text{Mn} \cdot \text{ATP}$ complex to wild-type and mutant EGF receptor cytosolic domain proteins

Solutions of either TKD61 (0.5 μM , $\circ$), TKD61-K/M (0.5 μM , ∇), or TKD61-D/A (1.0 μM , $\square$) and TNP-ATP [0.4 μM ($\circ$, ∇) or 0.1 μM ($\square$) in **A**; 2.0 μM ($\circ$), 4.0 μM (∇) or 1.0 μM ($\square$) in **B**] were titrated with increasing concentrations of ATP as the fluorescence of the nucleotide analogue was recorded. The displacement of TNP-ATP from the nucleotide binding site was indicated by an attenuation of the fluorescence intensity. Titrations were performed without (**A**) or with (**B**) the inclusion of 100 μM free MnCl_2 in the assay medium. Curve-fitting analysis yielded dissociation constants characterizing the binding of ATP and $\text{Mn} \cdot \text{ATP}$ to each of the TKD61 forms (see Table 1) and the theoretical titration curves shown [TKD61 and TKD-K/M (—); TKD61-D/A (---)].

while the defect in the Asp-813 $\rightarrow$ Ala mutant protein that abolishes catalytic activity did not significantly alter the nucleotide binding properties of the protein, the Lys-721 $\rightarrow$ Met mutation did perturb the nucleotide binding site, with a pronounced effect seen in the case of $\text{Mn} \cdot \text{ATP}$ binding. Although the CD spectra of the TKD61-K/M and TKD61-D/A mutant proteins indicated no gross perturbation of protein secondary structure relative to the wild-type protein, the intrinsic tryptophan fluores-

cence of the TKD61-K/M protein was attenuated. This latter finding suggested that the Lys-721 → Met mutation did alter the conformation of PTK catalytic domain.

The observed differences in the nucleotide binding properties of the Asp-813 → Ala and Lys-721 → Met mutant proteins, as was most evident in the binding of the divalent metal ion-nucleotide complex, correlate with observed differences in the signal transduction potentials of full-length receptor proteins incorporating these mutations [8,11]. The attenuated signal transduction capabilities of the Lys-721 → Met receptor mutant may reflect a significant perturbation of PTK structure, as was indicated here by an attenuation of intrinsic tryptophan fluorescence emission in TKD61-K/M, or may result from an abnormal enzyme/substrate interaction, as was evidenced in TKD61-K/M by a significant reduction in affinity for Mn·ATP and also by an attenuation of the fluorescence yield of enzyme-bound Mn·TNP-ATP (Figure 4B).

The results of this study are consistent with those of a previous investigation of the nucleotide binding properties of Lck, a non-receptor PTK in the Src family. In this investigation [27], a mutant Lck protein incorporating a Lys → Arg amino acid substitution at Lys-273, which corresponds to EGF receptor Lys-721, was found to be kinase-deficient. Also, the Lys-273 → Arg Lck mutant could be photoaffinity-labelled with 8-azido-ATP, which suggested that the defect in catalysis of this mutant was not in the binding of nucleotide, but in the enzymic process of phospho-transfer. However, nucleotide binding by the wild-type and mutant Lck proteins was not quantitatively analysed, so that changes in nucleotide binding affinity may not have been detected. In the case of the yeast cyclic AMP-dependent protein kinase, substitution of Ala for Lys-116, which also corresponds to EGF receptor Lys-721, greatly attenuates but does not abolish protein kinase activity. Although less than 1% of wild-type kinase activity was exhibited by the Lys-116 → Ala mutant enzyme, the K_m for Mg·ATP of this mutant could still be determined, and was found to be increased six-fold relative to the wild-type enzyme [28]. In the present study, the Lys-721 → Met substitution in the EGF receptor PTK induced similar effects: a near abolition of catalytic activity, and a large reduction (16-fold) in binding affinity for the bivalent metal ion-ATP substrate complex.

The recently reported three-dimensional structures of the fibroblast growth factor (FGF) receptor PTK domain with bound adenylyl diphosphonate [29] and the tris-phosphorylated insulin receptor PTK domain in a ternary complex with adenylyl imidodiphosphate and a Tyr-containing peptide substrate [30] both predict that substitution of conserved Lys-721 in the EGF receptor would perturb nucleotide binding. In the FGF receptor PTK domain structure, the homologue of Lys-721 appears to interact directly with both α - and β -phosphate oxygens of the bound nucleotide analogue; in the case of the insulin receptor PTK domain ternary complex, the corresponding Lys residue is hydrogen-bonded to an α -phosphate oxygen of the bound nucleotide. In contrast, neither structure shows a direct interaction between the homologue of EGF receptor Asp-813 and the bound nucleotide. In the structure of the insulin receptor PTK domain ternary complex, in which all phosphate oxygens of the nucleotide substrate are well ordered, the carboxyl group of this Asp is 0.58 nm (5.8 Å) from the nearest γ -phosphate oxygen. This Asp carboxyl group is however hydrogen-bonded to the hydroxyl group of the Tyr of the bound peptide substrate, which is consistent with its proposed role as a catalytic base. The observation that the Asp-813 → Ala substitution in the EGF receptor PTK domain did not perturb nucleotide binding is therefore consistent with the available structural data.

Robinson and co-workers [31] have recently analysed the structure and function of an ERK2 protein serine/threonine kinase incorporating a Lys → Arg amino acid substitution at a sequence position corresponding to conserved Lys-721 in the EGF receptor PTK. Whereas this mutant ERK2 protein displayed catalytic activity less than 5% of the wild-type protein, its K_m for ATP could again be determined and was found to be similar to that of the wild-type protein. Crystal structure comparisons of wild-type and mutant proteins indicated that no large alterations of protein secondary structure were induced by the amino acid substitution. However, analysis of the ATP complex of the mutant protein showed significant changes in the structure of the bound nucleotide relative to that of the wild-type protein. In contrast with the wild-type ERK2 protein, in which β - and γ -phosphate oxygens of bound ATP are co-ordinated with the Mg^{2+} cofactor, oxygens of all three phosphate moieties of the ATP substrate interact with Mg^{2+} in the active site of the Lys → Arg mutant protein. This and other orientational changes in the triphosphate group of bound ATP were apparently induced by a partial occlusion of the ATP binding site by the Arg residue.

Although the Lys → Met substitution analysed in the present work would not necessarily induce the same structural changes in the bound substrate molecule as were induced by the larger Arg residue in the case of ERK2, it is apparent that substitution of the conserved Lys in the protein kinase catalytic site can, without inducing significant protein structural changes, perturb the structure of the bound ATP molecule such that the rate of phospho-transfer to the peptide substrate is significantly reduced. Evidence for the perturbation of the conformation of the bound nucleotide was given in the case of the TKD61-K/M protein by the attenuation of the fluorescence of bound TNP-ATP and Mn·TNP-ATP complex (Figure 4).

In summary, we have characterized the effects that two well known kinase-inactivating mutations have on the nucleotide binding properties of the EGF receptor PTK. It is clear that EGF receptors harbouring either the Lys-721 → Met or Asp-813 → Ala substitution retained an ability to bind both ATP and a bivalent metal ion-ATP complex. In the case of the Lys-721 → Met substitution, the affinity of Mn·ATP binding was found to be significantly reduced relative to the wild-type protein. In contrast, the Asp-813 → Ala mutation, which also essentially abolished catalytic activity, had only minor effects on the nucleotide-binding properties of the enzyme. Hence, the principal effect of this mutation was assumed to be in the catalysis of the phospho-transfer reaction.

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