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Kinase receptor activation (KIRA): a rapid and accurate alternative to end-point bioassays[1]

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Abstract

We have developed a novel strategy for a rapid bioassay that is accurate, precise, sensitive, and high capacity. It is capable of quantifying ligand bioactivity by measuring ligand-induced receptor tyrosine kinase activation in terms of receptor phosphorylation. The assay, termed a 'kinase receptor activation' or KIRA, utilizes two separate microtiter plates, one for ligand stimulation of intact cells, and the other for receptor capture and phosphotyrosine ELISA. The assay makes use of either endogenously expressed receptors or stably transfected receptors with a polypeptide flag. KIRA assays for the ligands IGF-I and NGF were compared to their corresponding endpoint bioassays (3T3 cell proliferation for IGF-I and PC12 cell survival for NGF). The KIRA assays showed excellent correlation with the more classical endpoint bioassays. Further, they were highly reproducible, minimizing the requirement for repeat assays. The KIRA assay format has great potential as a rapid, accurate and precise bioassay, both for potency determination as well as stability-indicating analyses.

Keywords: Rapid bioassay; Ligand bioactivity; Receptor phosphorylation; Kinase receptor activation (KIRA)

1. Introduction

A bioassay is essentially any technique that assesses the bioactivity of a given ligand by measuring its induction of a response by an intact cell. Cellular response has generally implied proliferation, differentiation, enhanced survival, production/secretion of a product, cytotoxicity or perhaps self-deletion (apoptosis). But all of these cellular responses are the downstream events of a process that is, in fact, initiated by the stimulation of a receptor by its nominal ligand(s) (see Fig. 1). Phosphorylation of tyrosine residues upon ligand binding is a common mechanism of receptor tyrosine kinases (RTK) [1,2]. There are a number of

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RTK families. RTKs may be of the monomeric class I, III or IV families (including receptors for endothelial growth factor (EGF), platelet-derived growth factor (PDGF) or the neurotrophins, e.g. nerve growth factor or (NGF)). Alternatively, they may be of the tetrameric class II family (including the receptor for insulin-like growth factor-I (IGF-I)). For all four of the RTK families, binding of ligand results in the eventual phosphorylation of tyrosine residues within the cytoplasmic region of the receptor [2]. A similar situation exists for the serine/threonine kinase receptors (as typified by seven-transmembrane receptors) in that binding of the ligand to its receptor results in phosphorylation of serine and threonine residues. For all receptors, once phosphorylated, these residues serve as docking sites for adapter and/or intermediate proteins which, in turn, feed into the kinase cascade. This intermediate phosphorylation cascade allows amplification of the incoming signal as well as branching into specific pathways. These signals eventually lead to end-point activities such as survival, differentiation, etc. Viewed in this manner, all bioassays may be considered to be measures of ligand-induced receptor activation.

Any point along the course of interactions depicted in Fig. 1 may be utilized for the development of a bioassay. Traditional bioassays have been based on downstream events, such as cell proliferation or survival [3–6]. In the case of insulin-like growth factor I (IGF-I), the classical bioassays have been based on the IGF-I-induced proliferation of BALB/c 3T3 fibroblast cells [7–9] or MCF-7 human breast adenocarcinoma cells [10,11]. Nerve growth factor (NGF) bioactivity has traditionally been measured with assays based on the survival of or neurite outgrowth by rat PC12 cells [12,13]. However, there are problems inherent in the use of endpoint bioassays. Endpoint bioassays often suffer from high variability as well as long assay duration (3–6 days). Further, there is the possibility for the measured endpoint signal to be due to an alternative receptor that, when activated, leads to the same cellular phenotypic response (i.e. interference or cross-reactivity).

An alternative method that could more directly and specifically assess ligand bioactivity would be one based on the measurement of the initial commitment of a cell to respond, namely the initial activation of a specific receptor as evidenced by receptor phosphorylation. To this end, we have developed a high throughput, sensitive and quantitative 1-day assay capable of assessing receptor activation in terms of receptor phosphorylation. The assay, termed KIRA-ELISA ('kinase receptor activation', Fig. 2) employs a two-plate system, one 96-well cell culture plate for stimulation of intact cells, followed by receptor solublization and a second 96-well plate for receptor capture and analysis of tyrosine phosphorylation by ELISA [14,15]. Standard curve preparations and many samples may easily be run simultaneously in replicate and at several dilutions, readily allowing quantitation of ligand bioactivity in a large number of unknown samples.

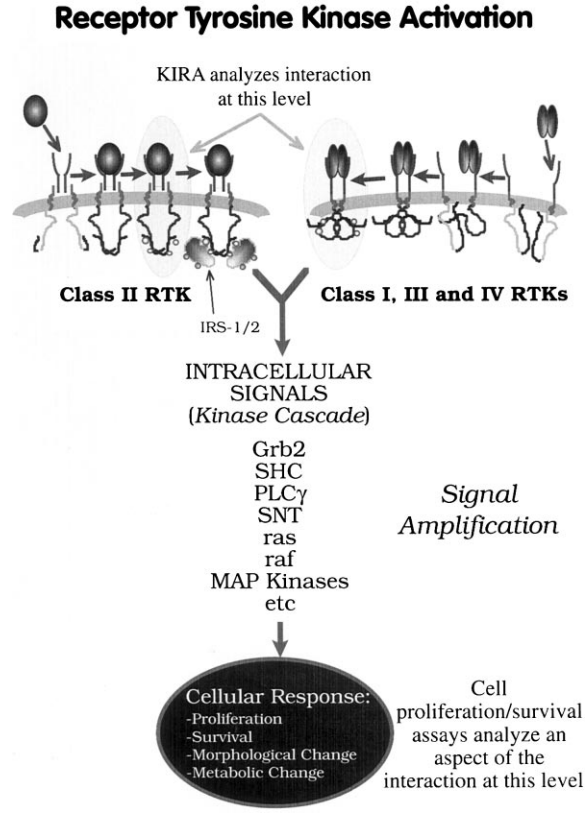

Fig. 1. Ligand activation of a receptor tyrosine kinase generates a signal that is maintained and amplified through the kinase cascade. The signal eventually results in a downstream end-point cellular response.

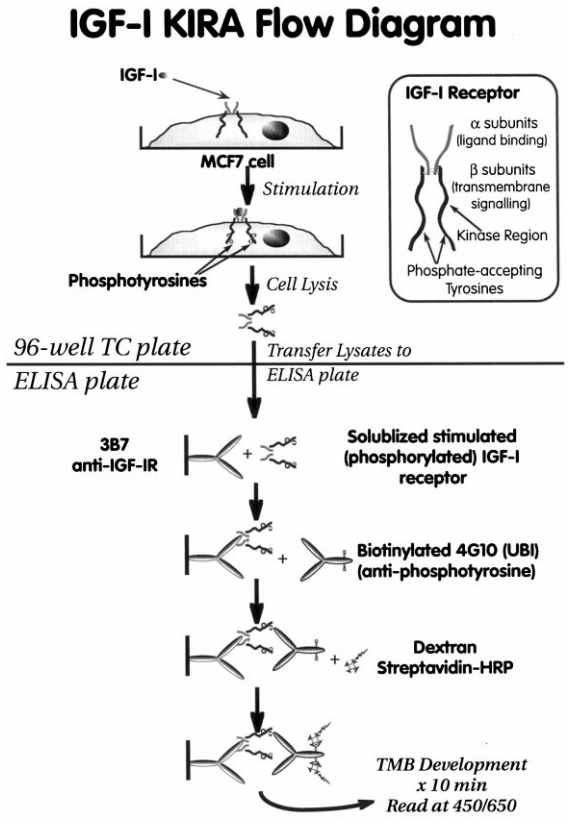

Fig. 2. Schematic diagram of the IGF-I KIRA.

2. Results and discussion

Two different approaches using the KIRA format will be described The first, using a cell line with an endogenous IGF-I receptor, was developed to measure the bioactivity of IGF-I. The second, using CHO cells stably transfected with a recombinant human trkA receptor with an N-terminal polypeptide flag (gD), was developed to measure the bioactivity of NGF [15].

2.1. IGF-I KIRA

The IGF-I KIRA utilizes adherent MCF-7 cells (from ATCC, Bethesda, MD) which are derived from a human breast adenocarcinoma. MCF-7 cells express endogenous IGF-I receptor (data not shown). For a detailed description of MCF-7 culture conditions and preparations, as well as detailed KIRA procedures and buffer constituents, please see Ref. [14].

To perform the IGF-I KIRA, MCF-7 cells were seeded in each well of a flat-bottomed 96-well culture plate (Falcon 3072, Lincoln Park, NJ) and cultured overnight (Fig. 2). The well supernatants were decanted, and the plates were lightly blotted on paper towels. Medium containing either experimental samples or the recombinant IGF-I standards were then added to each well. The cells were exposed to ligand for 15 min and the plates were decanted and blotted. Crude lysates were generated by addition to each well of lysis buffer containing Triton X-100 (Gibco/BRL, Grand Island, NY), sodium orthovanadate (to prevent dephosphorylation of the receptors), and a cocktail of protease inhibitors (ICN Biochemicals, Aurora, OH).

The crude lysates from the microtiter wells were transferred to an ELISA plate (Intermed, Denmark) that had been coated overnight with an IGF-I receptor- specific antibody (3B7 from Santa Cruz Biotech, Santa Cruz, CA), and 'blocked' with 0.5% BSA (Intergen, Purchase, NY) before use. The 3B7 antibody was used for coating at a concentration of 5.0 μg ml^{-1}. Incubation of the crude lysates in the ELISA plate effectively affinity purified the IGF-IR directly in the ELISA well. Following an extensive plate wash to removed unbound material (Skatron Instruments, Sterling, VIR), the degree of receptor tyrosine phosphorylation was quantified with biotinylated anti-phosphotyrosine monoclonal antibody (4G10, from Upstate Biological, Lake Placid, NY), followed by HRP-conjugated dextran–streptavidin (Amersham Pharmacy, Uppsala, Sweden). The degree of anti-phosphotyrosine binding was visualized with the development of a tetramethyl benzidine (TMB) substrate (Kerkegard and Perry, Gaithersberg, MD). The absorbance at 450 nm was read with a reference wavelength of 650 nm (A_{450}) (Molecular Devices, Paulo Alto, CA).

The standard curve shown in Fig. 3 was generated by averaging eight independent IGF-I KIRA assays. Each point is shown $\pm$S.E. Precision analyses (Table 1) of the IGF-I KIRA show that for high, mid and low controls, the intra- and inter-assay CVs were $\leq 11\%$.

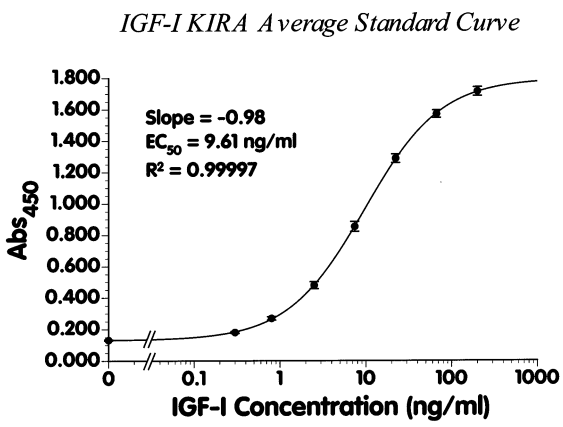

Fig. 3. IGF-I KIRA: averaged standard curve. Average of 13 individual runs (in duplicate) ± S.E.M. The standard curve is generated from recombinant human IGF-I at 200, 66.7, 22.2, 7.4, 2.5, 0.8, 0.3 and 0 ng/ml.

The IGF-I KIRA assay is highly specific for IGF-I (Fig. 4a). It showed no reactivity with the ligands human growth hormone (hGH), epithelial growth factor (EGF), thrombopoietin (TPO), relaxin, and TNFα. In further experiments, the IGF-I KIRA showed no cross-reactivity with the HER-2/HER-3 ligand, heregulin (data not shown). The assay demonstrated excellent specificity, even though we have detected receptors for several of these ligands on MCF-7 cells (specifically, EGF, TNFα and heregulin). The IGF-I KIRA does show cross-reactivity with insulin and

Table 1
Precision analysis in the IGF-I KIRA-ELISA assay

	Average concentration[a]	S.D.	%CV[b]
Intra-assay variation ($n = 24$)			
Low	1.10	0.10	7.20
Mid	5.30	0.30	5.00
High	22.30	2.10	9.50
Inter-assay variation ($n = 11$, each run value derived from three consecutive wells)			
Low	0.98	0.10	10.53
Mid	5.19	0.34	6.49
High	22.29	2.64	11.83

[a] Target values: high control, 25 ng/ml; mid control, 5.0 ng/ml; low control, 1.0 ng/ml.
[b] Intra-assay %CV determined as intra-assay SD/intra-assay average × 100.

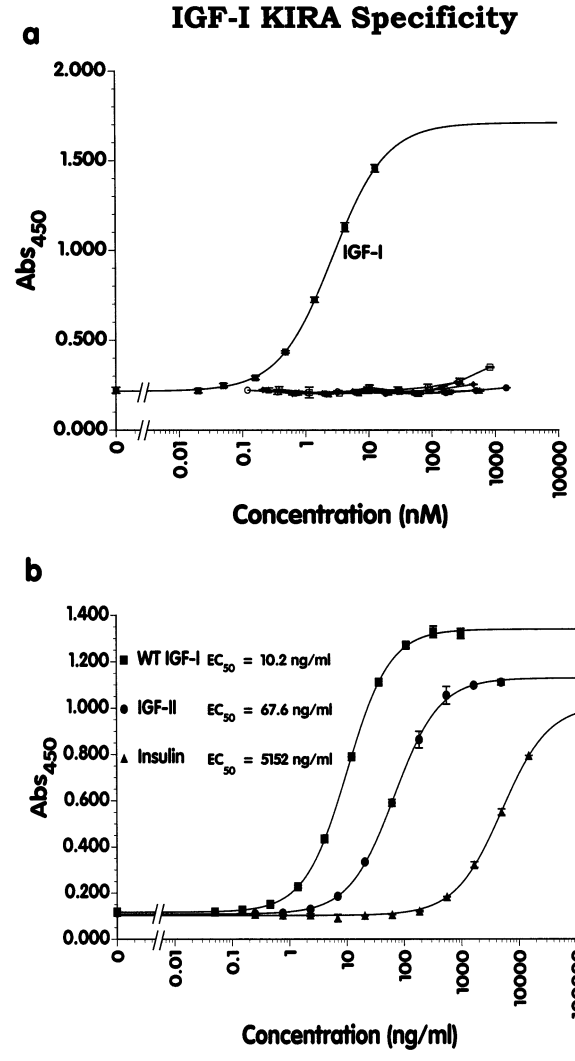

Fig. 4. IGF-I KIRA specificity test. (a) Non-related ligands. MCF-7 cells were stimulated with 0–20 nM IGF-I (■), or 0.1–1000 nM relaxin (●), TNFα (▲), hGH (◆), EGF (□) or TPO (○). Samples were run in triplicate and results are graphed as average $A_{450} \pm$ S.E.M. (b) IGF-I-related ligands. MCF-7 cells were stimulated with 0–800 ng/ml IGF-1 (■) or 0–10 μg/ml IGF-II (●) or insulin (▲). Samples were run in triplicate and results are graphed as average $A_{450} \pm$ S.E.M. EC_{50} values for each curve are shown.

IGF-II (Fig. 4b), but it has already been demonstrated that insulin and IGF-II will bind directly to the IGF-I receptor [16]. In agreement with the previously described data, the activity of IGF-II in the IGF-I KIRA is 7–8-fold less than IGF-I,

and that of insulin is more than 100-fold less. Further, the curve shapes of IGF-II and insulin stimulation are quite different from that of IGF-I stimulation, indicating that the kind of receptor activation induced by the cross-reactive ligands is different from that induced by the nominal ligand, IGF-I.

It was important to test the capacity of the IGF-I KIRA to work as a stability-indicating assay. Further, to show the validity of bioactivity values generated by the KIRA assay it was necessary to compare KIRA analyses to those done by classical endpoint bioassays. One of the endpoint bioassays used to measure IGF-I is the IGF-I induction of mouse 3T3 cell proliferation, quantified by incorporation of [^{3}H]thymidine or with metabolism-linked indicators such as tetrazolium salts (e.g. MTT) or Alamar Blue™ (AccuMed, Westlake, OH). We examined the variants produced during the biosynthesis, refolding and purification of recombinant IGF-I, and purified by neutral pH reversed-phase HPLC. Identified by conventional analytical characterization techniques, these variants included Met-sulfoxide, D-Ser, Glu-20, incorrectly folded (mixed disulfides), Des-Gly and des-Gly/Pro (missing the first or the first two N-terminal amino acids, respectively) IGF-I. We also tested des-Gly/Pro/Asp (des(1–3)) IGF-I, which, missing the three N-terminal amino acids, is incapable of binding any of the IGF-I binding proteins (IGF-I-BP). Both the 3T3 proliferation assay and the IGF-I KIRA demonstrated that all IGF-I variants except des(1–3) and incorrectly folded IGF-I have full bioactivity (Fig. 5a,b). Incorrectly folded IGF-I had approximately 10-fold less activity in both assays. There was, however, a difference between the two assay formats in the assignment of bioactivity to des(1–3) IGF-I. The data from the 3T3 proliferation assay suggest that des(1–3) IGF-I is at least twice as potent as the reference IGF-I. In reality, the reference IGF-I and the des(1–3) IGF-I should have the same specific bioactivity. The apparent increase in des(1–3) IGF-I bioactivity is, in fact, a decrease in an artifactual inhibition of IGF-I activity inherent in many bioassays. Many cell lines produce IGF-I-BPs capable of inhibiting IGF-I bioactivity. The des(1–3) IGF-I will not bind the IGF-I-BPs. The resulting absence of inhibition by indicator-cell-line-produced IGF-I-BPs will appear as an increase in des(1–3) IGF-I potency. Because the proliferative assay is a 3–5 day assay, it is very susceptible to interference by the 3T3-produced binding proteins. Since exposure to ligand in the KIRA assay is only for 15 min, there is much less time for the MCF-7 indicator cell line to produce IGF-I binding proteins to interfere

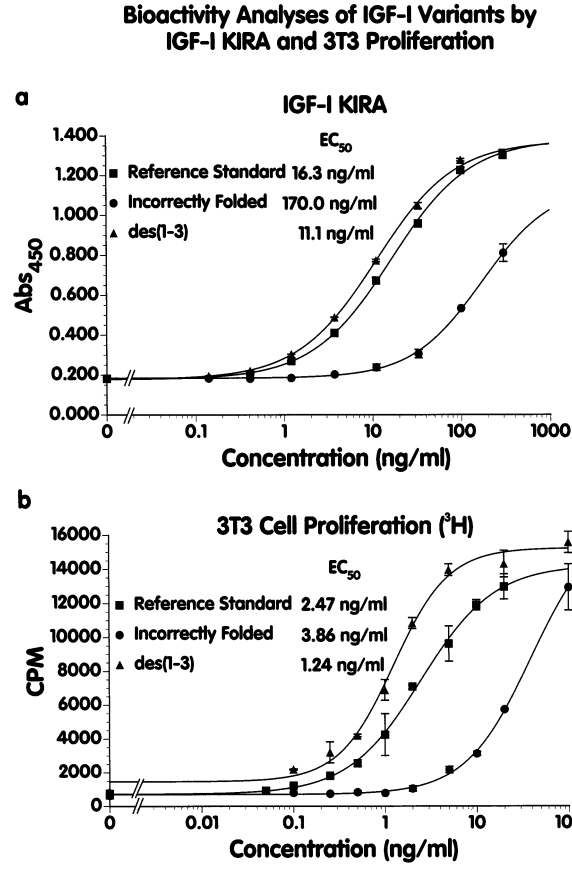

Fig. 5. Bioactivity analyses of IGF-I variants by IGF-I KIRA and 3T3 Cell proliferation. (a) IGF-I KIRA. MCF-7 cells were stimulated with 0–300 ng/ml IGF-I (■), incorrectly folded IGF-I (●) or des(1–3) IGF-I (▲). Samples were run in triplicate and results are graphed as average $A_{450} \pm$ S.E.M. EC_{50} values for each curve are shown. (b) 3T3 cell proliferation (^{3}H-thymidine uptake). BALB/c 3T3 cells were stimulated with 0–100 ng/ml IGF-I (■), incorrectly folded IGF-I (●) or des(1–3) IGF-I (▲). Samples were run in triplicate and results are graphed as average cpm ± S.E.M. EC_{50} values for each curve are shown.

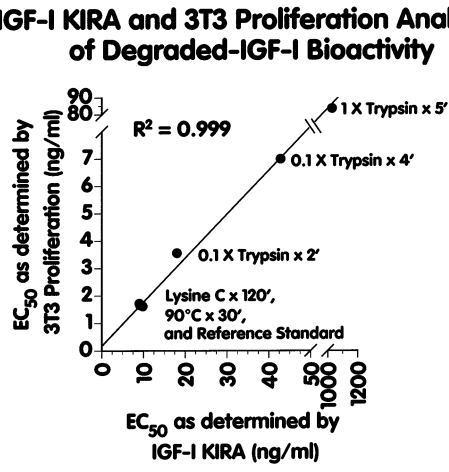

Fig. 6. Comparison between IGF-I KIRA and 3T3 cell proliferation analyses of degraded IGF-I bioactivity. The bioactivity of chemically, enzymatically or heat-degraded IGF-I samples were determined by both the IGF-I KIRA and the 3T3 cell proliferation assays. EC_{50} values for bioactivity were derived from both assays for each sample. The EC_{50} values derived from each are compared by paired-scatter analysis.

with the assay. Therefore, the apparent shift in potency by the des(1–3) IGF-I is much less in the KIRA than in the proliferative assay. However, the KIRA assay is sensitive to the action of IGF-I binding proteins. BP1 or BP3 added exogenously to the assay at equimolar ratios to added IGF-I results in a more than 80% reduction in signal (data not shown).

To more stringently test the correlation of the IGF-I KIRA and the 3T3 proliferation assay, a sample set of partially degraded IGF-I was generated using chemical or enzymatic treatment. IGF-I was incubated with dithiothreitol (DTT), lysine-C for 120 min, 1 × trypsin for 5 min or 0.1 × trypsin for 2 or 4 min. Alternatively, the IGF-I was heated at 90°C for 30 min. The samples were assayed by both IGF-I KIRA and by 3T3 proliferation. In both the proliferative assay and the KIRA the DTT-reduced IGF-I had no activity. For the remaining samples the EC_{50} values were determined in both bioassays for each sample and compared by paired scatter analysis (Fig. 6). The EC_{50} values generated by the two assays correlated well, with $R^2 = 0.999$ ($P < 0.0001$). Thus, we showed that not only does the IGF-I KIRA work as a stability-indicating assay, but the results obtained by measuring the initial cellular response correlate well with those measuring an endpoint response.

2.2. gD · trkA KIRA

Adherent cell lines with the appropriate endogenous receptor and antibodies specific for the receptor are not always readily available. Therefore, we developed a more universal format for the KIRA assay. To this end we stably transfected full-length recombinant human receptor into Chinese hamster ovary (CHO) cells. This allowed us to clone a polypeptide flag onto the N-terminus or C-terminus of the receptor. We used a 26-amino acid polypeptide derived from HSV glycoprotein D (gD), and as a capture reagent in the ELISA phase of the KIRA we used a monoclonal antibody specific for the gD flag (mAb 3C8) (Genentech). All KIRA assays based on this format used the 3C8 mAb as the capture antibody. The example given here is trkA, the receptor for the neurotrophin, NGF. The gD · trkA KIRA is described in detail elsewhere [15]. Essentially, the gD · trkA KIRA assay is performed identically to the IGF-I KIRA. The only differences are (1) the indicator cell (CHO cells); (2) the receptor (recombinant human trkA receptor); (3) an N-terminus gD-flag; and (4) mAb 3C8 as the capture antibody. All other reagents and all procedures are the same as described for the IGF-I KIRA.

It had been demonstrated by others that the trkA receptor was specific for NGF [17–19]. The gD · trkA KIRA showed the predicted ligand specificity. A strong signal was generated by NGF, but not by neurotrophin 4/5, brain-derived nerve growth factor, nor neurotrophin 3 [15]. The gD · trkA KIRA displayed excellent precision [15]. The intra- and inter-assay CVs for high, mid and low controls were all under 10%.

Two different sample sets were used to both test the gD · trkA KIRA as a stability indicating assay and compare the gD · trkA KIRA with the classical bioassay for NGF which is based upon the capacity of NGF to support the survival of neuron-like rat PC12 cells [13]. The first set (Fig. 7a) of NGF samples consisted of naturally occur-

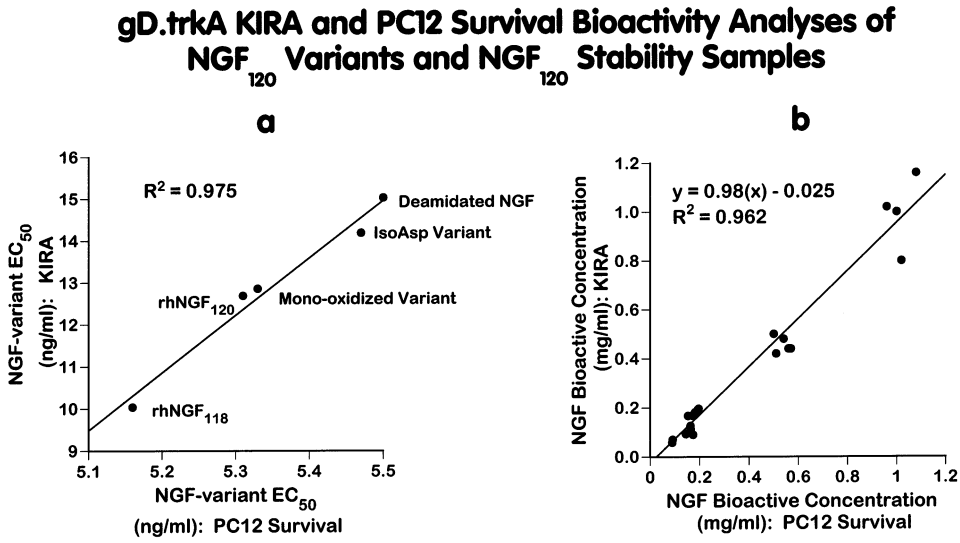

Fig. 7. Comparison between gD·trkA KIRA and PC12-survival analyses of NGF bioactivity. (a) NGF_{120} variants: EC_{50} comparisons. The bioactivities of $rhNGF_{118}$ and four variants, NGF_{120}, mono-oxidized NGF, IsoAsp NGF and deamidated NGF were determined by both gD·trkA KIRA and by PC12 survival assay. The EC_{50} values derived from each are compared by paired-scatter analysis. (b) NGF_{120} stability samples: quantitation of bioactive NGF. Bioactive NGF was quantified for NGF_{120} stability samples in the gD·trkA KIRA and the PC12 survival assay. The values shown have been corrected for assay dilution and represent the concentrations in the initial undiluted stability samples. The values derived from each assay are compared by paired-scatter analysis.

ring variants of the 120-amino acid form of human NGF (NGF_{120}), the initial product produced from the NGF mRNA transcript. The variants tested were wt NGF_{120}, deamidated NGF_{120} (at Asn^{45}), IsoAsp NGF_{120} (at Asp^{93}), mono-oxidized NGF_{120} (at Met^{37}) and a 118-amino acid form (NGF_{118}, the mature form that NGF_{120} is processed to in vivo with the loss of two C-terminal amino acids). The EC_{50} values from both assays for each sample were compared by paired scatter analysis. There was a good correlation between the KIRA and the PC12 survival assay with an $R^2 = 0.975$ ($P = 0.0017$).

The second set consisted of NGF_{120} stability samples; NGF_{120} stored in a variety of buffer formulations at several temperatures for various lengths of time (Fig. 7b). The buffers tested were acetate-, succinate-, histidine-, malate- and glutamate-based buffers at pH 4 or 5.5. The storage conditions examined were −70, 5, 25 and 37°C for 1–24 months. The bioactive NGF concentrations as determined by both assays (corrected for assay dilution) for each sample were compared by paired scatter analysis. Again, there was a good correlation between the KIRA and the PC12 survival assay with an $R^2 = 0.962$ ($P < 0.0001$). The slope was approximately 1.0, indicating that the KIRA and the PC12 assays measured NGF bioactivity equivalently.

In a test for accuracy of the KIRA format, we compared bioactivity (concentration) values derived from the gD·trkA KIRA to concentration values derived from a sandwich ELISA developed for NGF (Fig. 8). We used a set of plasma samples obtained from an NGF rat pharmacokinetic study. The results from the two assay formats correlated well, with an $R^2 = 0.92$ ($P < 0.0001$).

Taken together, the above data demonstrate that (1) the gD·trkA KIRA is comparable to the PC12 survival assay in evaluating relative bioactivity of NGF samples; thus (2) the gD·trkA is able to work as a stability indicating bioassay; (3) the gD·trkA KIRA and the PC12 survival assay are equivalent in quantifying absolute levels of bioactive NGF; and (4) the gD·trkA KIRA per-

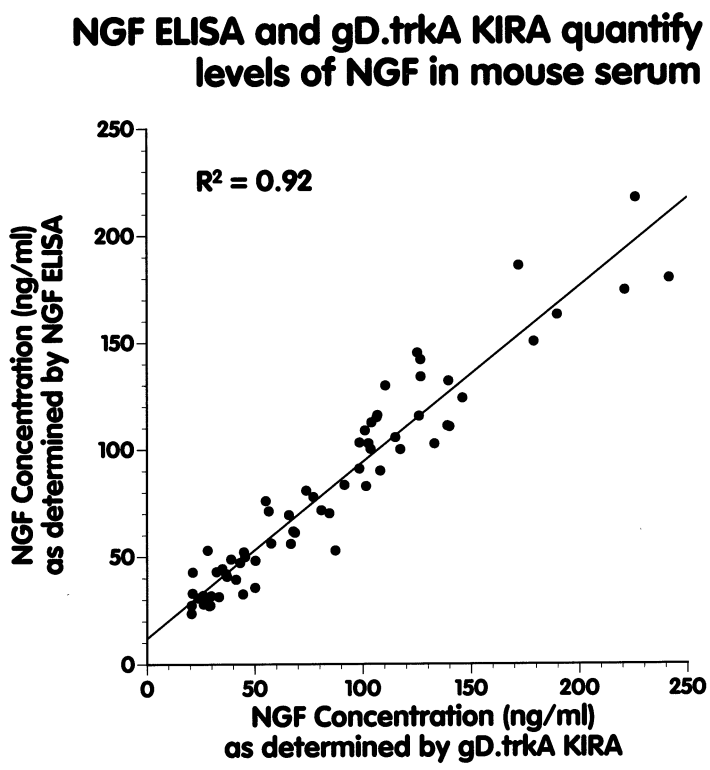

Fig. 8. Quantitation of rhNGF serum levels in treated rats: a comparison between gD·trk KIRA and ligand-specific sandwich ELISA. NGF levels in sera from rats receiving intravenous or subcutaneous administration of rhNGF were quantified by both gD·trkA KIRA and NGF ELISA. Values derived from gD·trkA KIRA are compared in a paired-scatter analysis with those derived from NGF ELISA.

forms with the accuracy of a sandwich ELISA assay.

3. Conclusions

The KIRA assay strategy provides a rapid and reproducible method to quantitatively assess ligand bioactivity. The assays described above demonstrated excellent correlation with more classical endpoint bioassays. The data from the IGF-I KIRA correlated very well with those from 3T3 cell proliferation and the data from the gD·trkA (NGF) KIRA correlated very well with those from the PC12 survival assay.

Further, the KIRA assay can be defined essentially as a monospecific assay in that it only evaluates the response of the receptor for which it is designed. The gD·trkA KIRA is highly specific for NGF. The IGF-I KIRA is specific for IGF-I or IGF-I-related molecules. Importantly, the IGF-I KIRA clearly shows that, although IGF-II and insulin do cross-react (which had been predicted), they do so with a markedly different response curve than the nominal ligand, IGF-I.

The KIRA assays may be performed with at least two different formats. One may use a cell line with an endogenous receptor (e.g. IGF-I) in conjunction with a receptor-specific capture antibody in the ELISA phase. Alternatively, as a more generalized format, one may use adherent cells stably transfected with a flagged receptor (e.g. gD·trk) in conjunction with a common 'capture' antibody (anti-gD).

It is potentially significant that the signal measured by the KIRA assay is upstream of a committed cellular response pathway. While this fact necessitates that the results from the KIRA analy-

ses be correlated with those from endpoint bioassays, it also means that the KIRA assay may have a more universal applicability. The cellular response measured by KIRA can be considered independent from any therapeutic indication for the ligand, and thus easily applicable to all therapeutic indications for that ligand.

There is a potential drawback to using the KIRA assay. Because the signal that is measured is upstream of the amplification provided by the kinase cascade, KIRA is usually about 10-fold less sensitive than end-point bioassays. However, for most process and product-release samples, high sensitivity is not an issue. Moreover, the benefits of the KIRA format far outweigh the loss of sensitivity. The KIRA assays are both precise and accurate. The intra- and inter-assay CVs for both the IGF-I and gD·trkA KIRA assays are, with few exceptions, $\leq 10\%$. When one considers that the KIRA assay is actually both a bioassay and an ELISA, this level of precision is quite remarkable. Evidence for accuracy of the KIRA assay is shown by the similar quantitation of NGF samples by both NGF KIRA and sandwich NGF ELISA. Finally, as the ELISA segment of all versions of the KIRA assays use the same reagents and conditions, the assay is easily adapted to automation. The KIRA assay format certainly has the capacity to be used as either a potency assay or as a stability-indicating assay

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