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Detection of neu differentiation factor with a biospecific affinity sensor during chromatography

Hsieng S. Lu*, David Chang, David Brankow, Duanzhi Wen

Amgen Inc., Amgen Center, Thousand Oaks, CA 91320, USA

Abstract

A technique using a biospecific affinity sensor, BIAcore, was applied to monitor and determine mammalian cell-derived neu differentiation factor (NDF) in column fractions during chromatography. Specific purified polyclonal antibody against *Escherichia coli*-derived NDF was chemically bound to the surface of BIAcore sensor chips and the derivatized sensor chips were used to detect the specific binding of NDF. The measurement of NDF at very low levels can be assessed by injecting small volumes of the crude media or column fractions into the BIAcore sensor containing antibody-bound sensor chips. This automated procedure performed under computer programming control allows direct measurement of multiple NDF samples in a short period of time and provides excellent quantitative data, which is not possible using other related methods such as Western blotting, sodium dodecyl sulfate–polyacrylamide gel electrophoresis and stimulatory activity assay on receptor autophosphorylation.

1. Introduction

The neu differentiation factor (NDF) or heregulin gene family encodes a number of secreted forms of soluble factors that specifically interact with an M_r 185 000 tyrosine kinase receptor, termed $p185^{neu}$, HER-2 or c-erbB-2 [1–4]. Upon ligand-receptor binding, NDF can stimulate receptor autophosphorylation and promote subsequent cellular functions [2,4]. Overexpression of $p185^{neu}$ or HER-2 has been found in some neoplastic tissues and also associated with poor prognosis for several types of cancers, including breast cancer [5–8]. The structural and functional aspects of the multiplicity of the human and rat NDF have been investigated [3,4].

Our efforts, aiming at understanding the structural characteristics of soluble NDF forms and their roles in as yet unknown biological functions, led to the production and isolation of rat NDF from rat cell lines in addition to human and rat NDF recombinant forms in Chinese hamster ovary (CHO) cells. As both natural cell lines and the engineered CHO cells can only secrete a small amount of NDF, the detection and isolation of mammalian cell-derived NDF has been difficult [1,9,10]. Although Western blotting, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and tyrosine kinase phosphorylation assay [9] can be adapted to monitor the column effluents during fractionation, these methods, in general, are time consuming, less sensitive and not applicable to samples containing low expression levels of NDF. In this paper, we describe the use of an antibody-based bioaffinity technique (BIAcore) [11,12] as an alternative to measure rat NDF

* Corresponding author.

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during column chromatography from media conditioned by natural cell line or by recombinant CHO cells. This automated procedure is capable of handling multiple column fractions with sufficient speed and sensitivity in detection and provides precise NDF quantification for various samples including pooled fractions and purified NDF preparation.

2. Experimental

2.1. Production and isolation of NDF

NDF prepared from rat-1 EJ cells was produced according to our previous papers [1,2]. Expression of rat proNDF-α2 and -β4 forms (encoding 639 and 661 amino acid proNDFs, respectively) in CHO cells was also described in previous papers [4,10]. In the cultured medium, the NDF proteins are secreted as M_r 44 000 soluble molecules. They were isolated by a series of purification steps including concentration of medium by diafiltration and chromatography using heparin-Sepharose, DEAE-Sepharose and phenyl-Sepharose columns [10]. Western blotting and SDS-PAGE were performed according to previously described methods [10].

2.2. Polyclonal antiserum production

Rabbit polyclonal anti-rate NDF antibody was produced against *Escherichia coli*-derived recombinant rat NDF-α2 and purified using an Affigel affinity column coupled with the same rat NDF protein. The partially purified antibody was used in assays with a BIAcore system or Western blotting.

2.3. Real-time biospecific interaction analysis (BIA)

The analysis was performed using a BIAcore instrument (Pharmacia Biosensor, Uppsala, Sweden) [11,12]. To detect the specific binding between rat NDF and the antibody, a sensor chip surface used in the BIAcore system was first immobilized with affinity-purified rabbit antibody against *E. coli*-derived rat NDF-α2. The immobilization was performed using sensor chip CM5 programs according to the procedures described in the instructions of the amine coupling kit (Pharmacia). Samples containing various amounts of NDF were diluted with BIAcore HBS buffer [10 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4)–150 m*M* NaCl–3.4 m*M* EDTA and 0.05% BIAcore surfactant P20] to a concentration ranging from 5 to 300 ng/ml before injection. Samples were automatically injected and flowed through the sensor chip surface. Injection volumes ranged from 10 to 100 μl depending on sample concentration. After sample injection, the chip surface was washed with HBS buffer to eliminate non-specific binding. Any NDF bound to the surface results in an increase in surface plasma resonance (SPR), which can be detected optically by the equipment. The intensity of this resonance response, expressed as resonance units (RU), is proportional to the amount of NDF bound to the chip surface. After calibration with a standard amount of CHO cell-derived rat NDF-α2 which was determined by amino acid analysis [10], samples containing unknown NDF concentrations can be analysed.

Regeneration of the NDF-bound sensor chips was obtained by a washing step with 10 m*M* HCl (pH 2.2) and an equilibration step with HBS buffer. The regenerated sensor chips can then be used to assay the next sample.

3. Results and discussion

Fig. 1A shows SPR raw data obtained from the analysis of an NDF standard produced in engineered *E. coli* cells using a freshly prepared antibody-bound sensor chip. In these particular analyses, the BIAcore program used a 12-min run time, including a 5-min sample analysis (as shown) and an extra 7-min HCl washing and equilibration (not shown). The SPR was obtained from the RU difference between the sample injection starting point (S) and the equilibration point after sample injection (E). Sensorgram 1 as indicated in Fig. 1A represents a blank run containing only sample buffer and

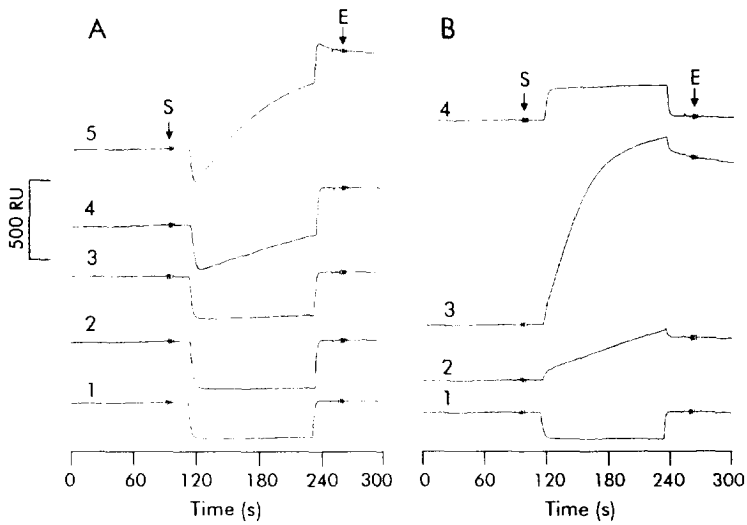


Fig. 1. Real-time BIAcore assay of rat NDF using a sensor chip coupled with anti-NDF antibody. (A) Sensorgrams from analysis of an NDF standard; (B) sensorgrams from analysis of fractions from heparose-Sepharose chromatography (see Fig. 3).

shows almost no response (RU = 5). Sensorgrams 2–5 represent analyses of NDF standard at concentrations of 5, 20, 100 and 300 ng/ml. The background RU for controls ranges from ca. −10 to +10 RU. The data clearly show that the method can detect NDF at concentrations above 20 ng/ml. Samples were always diluted with HBS buffer (minimum dilution = 1:1). Although concentrated culture media can be analysed directly they can potentially cause background interference due to light scattering.

Fig. 2 illustrates typical titration curves for standard rat NDF-α-2 and -β4 recombinantly produced in CHO cells as well as rat NDF-α2 produced in *E. coli*. These data indicate that antibody produced against bacterially derived rat NDF-α2 is able to recognize glycosylated forms of rat NDF-α2 and -β4 derived from CHO cells [10], but with a lesser extent of binding affinity. This lower binding for CHO-derived NDFs may be contributed to by carbohydrates present in the molecule. The difference in binding between CHO-derived NDF-α2 and -β4 may be due to variations in the primary structure at the EGF domains and their C-termini. The difference may also be caused by the pattern of glycosylation, as CHO cell-derived rat NDF-α2 and -β4 contain two Asn-linked and eleven O-linked sugars [10]. Although the responses are different among these NDF forms, the BIAcore response versus NDF concentration is linear at all samples, indicating that the antibody coupled to the sensor chips is capable of recognizing both *E. coli* and CHO cell-derived NDF feeding into the sensor surface in a concentration-dependent manner. The NDF concentrations that fall into the linear quantitation range is approximately 10–200 ng/ml for rat NDF-α2 derived from *E. coli*, 20–300 ng/ml for CHO-derived NDF-α2 and 50–600 ng/ml for CHO-derived NDF-β4.

Precautions should be taken into consideration when the bioaffinity technique is used for sample monitoring and quantification. For example, the accuracy of measurement relies on the recognition of NDF by the coupled antibody being within a linear response range (see Fig. 2); samples containing NDF above the concentration limit require further dilutions with HBS buffer. A gradual decrease in RU occurred on daily use. To compensate for this problem, calibration standards are always established at the beginning and end of runs to ensure consistency of the results within 24-h operation. A 3–5% variation for the observed RU is acceptable and is frequently observed within 24 h. After coupling to the sensor chips, usually anti-

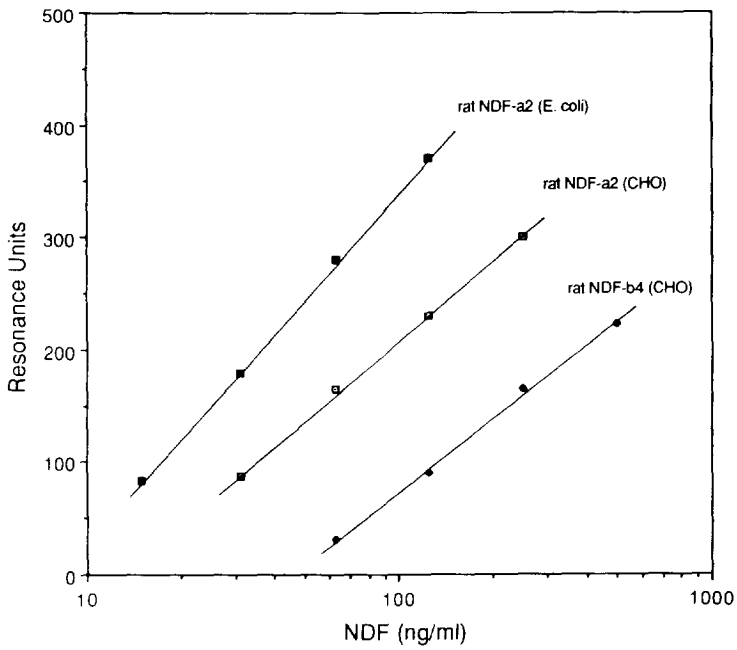


Fig. 2. Calibration graphs for determination of CHO cell-derived recombinant rat NDF-α2 and -β4 in solution versus rat NDF-α2 produced in *E. coli*. The resonance response on the vertical axis is proportional to the amount of the NDF bound to anti-NDF antibody on the sensor chip surface.

bodies are stable and can be used for approximately 2 weeks, at which time the resonance unit may remain less than half of a freshly prepared chip. Chips coupled with antibodies can be repeatedly assayed for approximately 1000 samples. Thus, as a routine and automatic operation, this procedure can continuously assess measurements for 2 weeks using a single chip. However, chips coupled with other protein molecules, especially soluble receptors, may be less stable, and might have a much shorter life span. This decreased SPR or shorter life span may be partly due to washout or acid-induced inactivation of the bound antibodies or proteins during regeneration. The coupling efficiency is usually reproducible with antibodies obtained from the same purification batch. However, significant variations in resonance response did occur using different batches of antibodies. Coupling of lower titer antibodies sometimes yielded an unusable chip.

As shown in Table 1, the linear response characteristic for binding between NDF and antibody allows the reliable quantification of crude NDF preparation concentrated from the cultured medium. The accurate quantification of NDF was not possible by methods using both Western blotting and tyrosine kinase assays [1,2,10]. SDS-PAGE gave multiple overlapping protein bands from the concentrated medium over a wide range of molecular mass, and the method was unable to locate the M_r 40 000–44 000 NDF band after Coomassie Blue staining in samples having low NDF expression.

Fig. 1B shows SPR raw data obtained from analyses of several column fractions from heparin-Sepharose chromatography of rat NDF-α2 produced in CHO cells. In these analyses, fractions were diluted 50–100-fold with HBS buffer before sample loading. Sensorgrams 1–4 are for samples taken from fractions 20, 53, 60 and 80 as indicated in Fig. 3A. Fraction 60 clearly gave a very high reading (RU > 600), indicating that it exceeds the linear response range. Any samples such as this were diluted further and reanalyzed within the linear response

Table 1
Determination of rat NDF by 280 nm and BIAcore detection

Step	Volume (ml)	Protein[a] (280 nm)	NDF[b] (mg)	Purification (-fold)	Yield (%)
(A) CHO cell-derived rat NDF-β4					
(1) Medium	10000	–	–	–	–
(2) Concentrate	1000	1600.0	5.52	1	100
(3) Heparin-Sepharose	110	22.2	3.60	72	65.2
(4) DEAE-Sepharose	78	10.8	2.50	148	45.3
(5) Phenyl-Sepharose and concentration	7	1.10	2.00	1455	36.2
(B) Rat-1 EJ cell-derived NDF					
(1) Medium	115000	–	–	–	–
(2) Concentration	2100	10710.0	0.081	1	100
(3) Heparin-Sepharose	165	49.5	0.042	216	52.9
(4) DEAE-Sepharose	60	3.5	0.028	3060	35.0
(5) Phenyl-Sepharose and concentration	1.7	~0.015[c]	0.025	–[c]	31.3

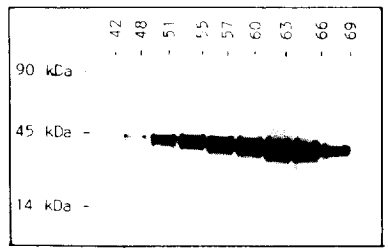
For detailed purification procedure, see Experimental.
[a] Proteins were measured by absorbance at 280 nm. The numbers on this column are the total absorbance readings.
[b] The NDF content was measured by BIAcore (see Experimental).
[c] The 280-nm absorbance is not accurate owing to low concentration, hence the purification fold cannot be precisely calculated.

range. Fig. 3A shows a typical chromatographic profile of the first heparin-Sepharose column step to purify rat NDF-β4 from 1 l of concentrated medium (see also Table 1A). The chromatographic profile shows both the protein elution detected by the 280 nm absorption and BIAcore quantification. The amount of NDF eluted was quantified and expressed in μg per fraction after the detected resonance units were calibrated with a calibration graph (Fig. 2). Clearly, the NDF eluted later as a shoulder by 280-nm measurement and was located at the right portion of the major protein peak by BIAcore detection. This observation matches the detected M_r 44 000 NDF bands observed in Western blots (inset in Fig. 3) where the antibody reacts strongly with the SDS-denatured NDF protein bands After SDS-PAGE.

The NDF elution profile detected by antibody surface binding in BIAcore also works really well for both DEAE- and phenyl-Sepharose chromatography. In these chromatographic separations, detection at 280 nm only picks up NDF protein peak at very low absorption. As shown in Fig. 2B, BIAcore clearly demonstrates its better sensitivity and capability of identifying the NDF fractions during phenyl-Sepharose chromatography. The sensitivity limit for NDF quantification is approximately 20–30 ng/ml.

Table 1A summarizes the purification recovery and step yield for recombinant NDF-β4 determined by absorption measurement and BIAcore. The overall step yield for the recovery of NDF protein is about 36% when measured by BIAcore. Various chromatographic steps yielded approximately 1500-fold purification from the concentrated medium. The NDF content in the final concentrated and purified preparation is calculated to be 2 mg, consistent with the data precisely quantified by amino acid analysis.

Rat-1 EJ cells produce only very small amounts (700 ng/l) of natural NDF. The detection of the NDF fraction using phosphorylation assay has been difficult [1,2]. Assay using BIAcore allowed high-sensitivity detection and quantification of NDF. As shown in Table 1B, NDF can only be detected after substantial concentration. A 2.1-l diafiltrate concentrated from 115 l of medium contained less than 100 μg of NDF. Following similar purification proce-



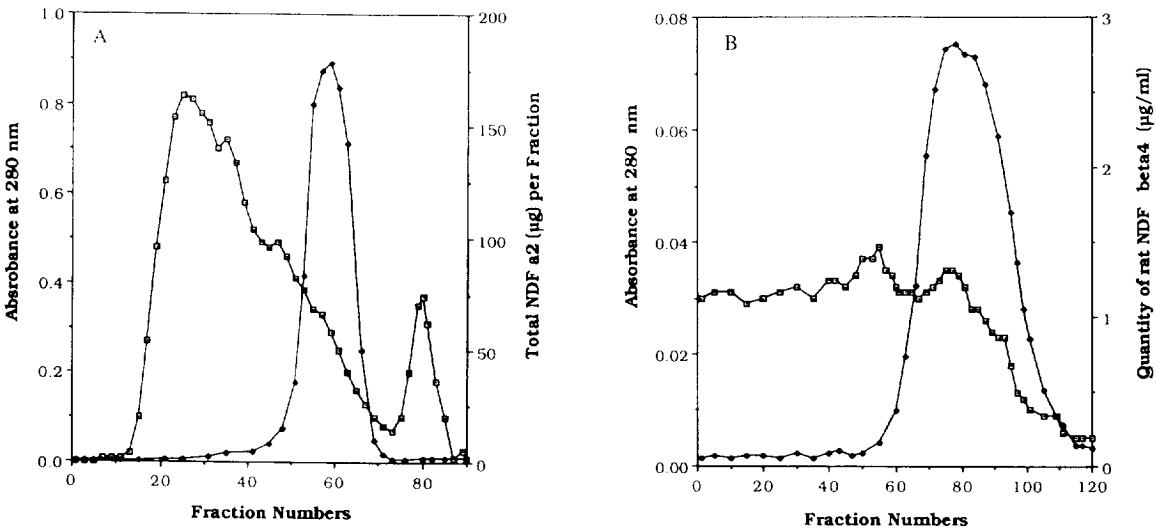


Fig. 3. (A) Heparin-Sepharose chromatography of a crude medium containing recombinant rat NDF-β4 (also see Table 1). The concentrated medium loaded on to the column (20 × 2.5 cm I.D.) contains 5.52 mg of NDF and 1.6 g of total proteins. After sample loading, 95% of contaminating proteins was washed off the column by 0.3 *M* NaCl in PBS buffer (pH 7.2). The column was then eluted with a 0.3–1.0 *M* NaCl linear gradient to fractionate NDF. Fractions (3.0 ml) were collected and detected for 280 nm absorption (□). Aliquots of fractions, after dilution, were also subjected to BIAcore analysis (◆). Inset: Western blotting of fractions shown in (A). (B) Phenyl-Sepharose chromatography of a DEAE-Sepharose pool containing recombinant rat NDF-β4 (also see Table 1). A linear gradient was established from 1.5 to 0 *M* ammonium sulfate in PBS (150 ml each) after sample loading. Fractions (1.8 ml) were collected and subjected to UV absorption measurement (□) and BIAcore assay (◆). kDa = kilodalton.

dures, separation of NDF can be obtained. Fig. 4 illustrates the DEAE-Sepharose chromatographic profile of a natural rat NDF preparation after a heparin-Sepharose column fractionation. A small shoulder peak, detected at 280 nm and eluted earlier than a major contaminating peak, was recognized to contain NDF activity. After final purification, a total of 25 μg of pure NDF was obtained.

The above results support the advantage of BIAcore analysis over phosphorylation assay and Western blotting in sample detection and NDF quantification. As the tyrosine kinase stimulatory activity of NDF was performed using membrane-bound $p185^{neu}$ in whole cell lysates, the assay results displayed phosphorylated receptor in protein blots which was visualized by anti-phosphotyrosine antibody after NDF stimulation. As the assay requires several manual operations, the observed data would inevitably vary from run to run. The phosphorylation assay is thus less applicable in handling multiple chromatographic samples and performing precise quantification. Western blotting is also inherently not suitable

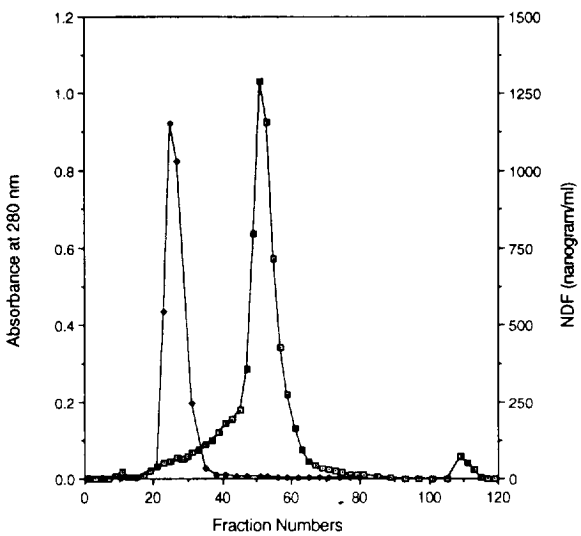


Fig. 4. DEAE-Sepharose chromatography of an NDF sample obtained from heparin-Sepharose chromatography of concentrated media conditioned by rat-1 EJ cells (see Table 1B). A linear gradient from 0 to 0.5 *M* NaCl in 10 m*M* phosphate buffer (pH 7.0) (200 ml each) was applied after loading. Fractions (3 ml) were collected and assayed by 280-nm absorption measurement (□) and BIAcore assay (◆).

for quantification as recognition of a target protein by its specific antibody is performed by an SDS-PAGE–electroblotting procedure.

In summary, the specific bioaffinity technique provides an alternative measurement for the quantification of NDF produced naturally or recombinantly during chromatographic fractionation. This automated method provides sufficient sensitivity for detection and quantification and should be applicable to other proteins using similar antibody binding as described here and other affinity assay techniques such as receptor–ligand binding. A recent paper has reported the use of such an approach to discover the ligand for an ECK receptor of unknown function [13].

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