FEBS Letters 580 (2006) 3386–3390

The neural cell adhesion molecule binds to fibroblast growth factor receptor 2

Claus Christensen, Jes B. Lauridsen, Vladimir Berezin, Elisabeth Bock, Vladislav V. Kiselyov*

Protein Laboratory, Copenhagen University, Blegdamsvej 3C, bld. 6.2, DK-2200, Copenhagen, Denmark

Received 10 March 2006; revised 26 April 2006; accepted 3 May 2006

Available online 11 May 2006

Edited by Miguel De la Rosa

Abstract The neural cell adhesion molecule (NCAM) can bind to and activate fibroblast growth factor receptor 1 (FGFR1). However, there are four major FGFR isoforms (FGFR1–FGFR4), and it is not known whether NCAM also interacts directly with the other three FGFR isoforms. In this study, we show by surface plasmon resonance analysis that NCAM can bind to FGFR2 with an affinity similar to that for the NCAM–FGFR1 interaction. However, the kinetic parameters for the NCAM–FGFR2 binding are different from those of the NCAM–FGFR1 binding. Both receptors were shown to cycle relatively fast between the NCAM bound and unbound states, although FGFR2 cycling was clearly faster (13 times) than the FGFR1 cycling. Moreover, ATP was more effective in inhibiting the binding of NCAM to FGFR1 than to FGFR2, indicating that the binding sites in NCAM for the two receptors are similar, but not identical.

Keywords: Neural cell adhesion molecule; Fibroblast growth factor receptor; Binding; Interaction; Surface plasmon resonance

1. Introduction

The fibroblast growth factor receptors (FGFR1–FGFR4) are a family of transmembrane tyrosine kinases involved in signalling via interactions with the family of fibroblast growth factors (FGF1–FGF23) [5]. FGFRs regulate a multitude of cellular processes including cell growth, differentiation, migration and survival, and have been implicated in a number of physiological and pathological processes including angiogenesis, wound healing and cancer. The prototypical FGFR consists of three immunoglobulin (Ig) modules (Ig1–Ig3), a transmembrane helix and a cytoplasmic tyrosine kinase domain. FGF-FGFR binding results in the FGFR dimerization leading to auto-phosphorylation of the receptor tyrosine kinase domains [10]. The FGFR–ligand interaction is mediated by the Ig2 and Ig3 modules, while the Ig1 module is thought to have a regulatory function.

FGFR1 can also be activated by cell adhesion molecules such as L1, N-cadherin and the neural cell adhesion molecule (NCAM). NCAM belongs to the Ig superfamily and consists of five extracellular Ig modules, two fibronectin type III (F3) modules and a cytoplasmic part of varying length [1]. NCAM mediates cell–cell and cell–substratum adhesion by means of homophilic binding and numerous heterophilic interactions [11]. The homophilic interaction mediated by NCAM leads to activation of FGFR1, which in turn results in a context-dependent biological response, such as induction of axonal growth during development, as well as modulation of synaptic plasticity. The structural determinants for the NCAM–FGFR1 interaction have recently been characterized [6,7]. It was shown by surface plasmon resonance (SPR) analysis that an NCAM fragment consisting of the first and second F3 modules bound to the Ig2–Ig3 modules of the FGFR1 (3C subtype) with a dissociation constant (K_d) of 10 μM. The NCAM–FGFR1 interaction site in NCAM was further mapped by nuclear magnetic resonance (NMR) analysis to the FG loop region of the second F3 module, and a peptide corresponding to this region (termed FGL) was capable of binding to the FGFR1 Ig modules 2 and 3 [6]. The FGL peptide also has been shown to induce neurite outgrowth in primary neurons and promote neuronal cell survival in vitro and in vivo [8,9] The FG loop region of the second F3 module of NCAM contains an ATP binding motif and is thought to be responsible for the ATPase activity of NCAM. The function of the ATPase activity of NCAM is not fully understood. However, it was shown by NMR that the second F3 module of NCAM binds ATP via its ATP binding motif, and by SPR it was shown that ATP can inhibit the NCAM–FGFR1 interaction, thus suggesting that ATP may be a regulator of the NCAM–FGFR1 interaction. This notion is further supported by the fact that ATP inhibits NCAM-mediated neurite outgrowth [6]. Since there are four major FGFR isoforms, it is of interest to test if NCAM also can bind to the other isoforms, FGFR2 in particular. A number of identified missense mutations in FGFR2 has been shown to lead to craniofacial pathology due to premature fusion of cranial sutures, a hallmark of over 100 distinct syndromes, including Apert, Pfeiffer and Crouzon syndromes [3].

Here we show by SPR that the combined first and second F3 modules of NCAM and the FGL peptide bind to the combined second and third Ig modules of FGFR2 (3C subtype) with K_d values of 9.21 and 1.75 μM, respectively, and that ATP is able to inhibit these interactions. Thus, the affinity of the NCAM–FGFR2 interaction appears to be similar to that of the NCAM–FGFR1 interaction. Based on this, we propose that NCAM can activate FGFR2. However, although both receptors were shown to cycle relatively fast between the NCAM bound and unbound state, FGFR2 cycling was clearly faster

*Corresponding author. Fax: +45 35 36 01 16.
E-mail address: vk@plab.ku.dk (V.V. Kiselyov).

0014-5793/$32.00
doi:10.1016/j.febslet.2006.05.008

than that of FGFR1, and ATP was more effectively inhibiting NCAM binding to FGFR1 than to FGFR2.

2. Results

In order to study the NCAM–FGFR2 interaction, we used recombinant proteins consisting of the NCAM F3 modules 1–2 [6], and the FGFR2 (3C subtype) Ig modules 2–3. Both recombinant proteins were expressed in a yeast expression system of *Pichia pastoris*. To test if the FGFR2 fragment was expressed in a functionally active form, the protein was immobilized on the surface of a CM-5 sensor chip, and its binding to FGF1 was studied by SPR and compared to that of the corresponding FGFR1 fragment, which previously had been expressed in *Drosophila* cells and by NMR analysis was shown to be properly folded [6]. The binding curves for the FGFR1-FGF1 and FGFR2-FGF1 interactions are shown in Fig. 1. The K_d values for both interactions were estimated to be approximately 5 nM, which is similar to the K_d values for this interaction obtained by other researchers using SPR [4]. Thus, the immobilized FGFR2 fragment appears to be functional and suitable for further analysis.

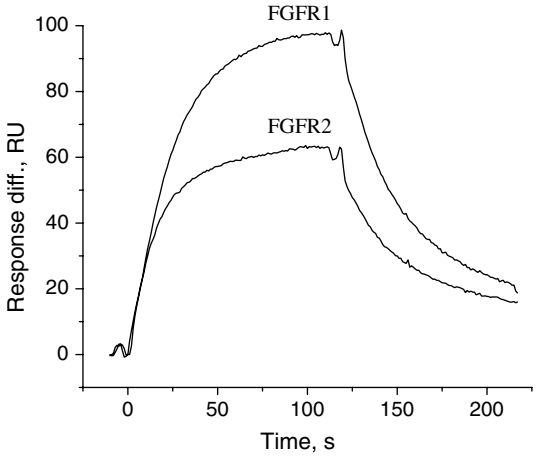

Fig. 1. Binding of FGF1 to the FGFR1 and FGFR2 Ig modules 2–3. FGF1 concentration was 100 nM.

2.1. The NCAM F3 modules 1–2 and the FGL peptide bind to the FGFR2 Ig modules 2–3

Since it previously had been shown that the NCAM F3 modules 1–2 bound to the FGFR1 Ig modules 2–3 [6], it was of interest to test if this interaction was similar to that of NCAM and FGFR2. Therefore, binding of the immobilized FGFR2 Ig modules 2–3 to soluble NCAM F3 modules 1–2 was studied by SPR (Fig. 2A). In order to determine the K_d value of the NCAM–FGFR2 interaction, the equilibrium binding level of the F3 modules was plotted versus the concentration of the F3 modules in solution and fitted with an equation describing the single-site receptor–ligand equilibrium binding (Fig. 2B).

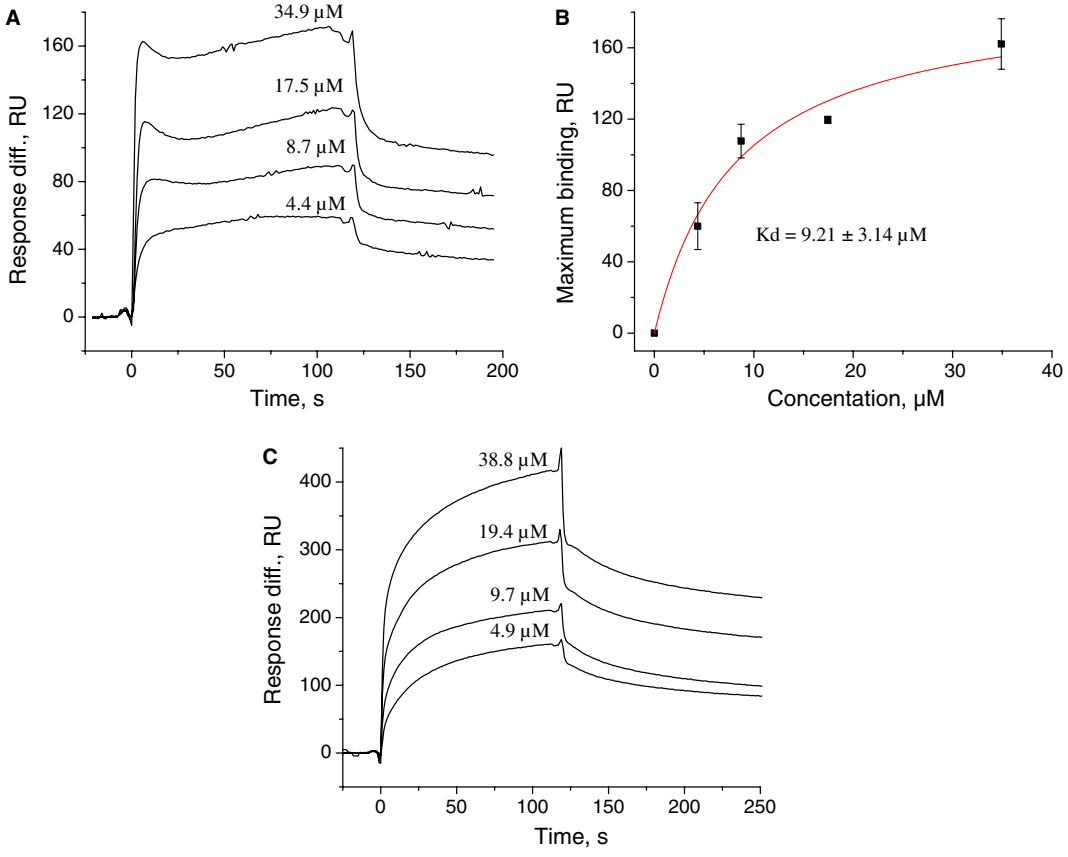

Fig. 2. Binding of the NCAM F3 modules 1–2 and the FGL peptide to the FGFR2 Ig modules 2–3. (A) Binding of the NCAM fragment, and (C) binding of the FGL peptide at the specified concentrations. (B) The equilibrium binding level of the NCAM fragment versus the fragment's concentration and the fitted curve.

The equilibrium binding analysis method was chosen instead of fitting the individual association and dissociation phases because the association phase (Fig. 2A) was atypical. The coefficient of dissociation rate (k_d) was determined by fitting the dissociation phase with the corresponding equation, and the coefficient of association rate (k_a) was estimated by dividing k_d with K_d. In comparison to the NCAM–FGFR1 binding, the NCAM–FGFR2 binding is characterized by much faster association and dissociation phases and approximately the same affinity. A detailed comparison of the kinetic parameters for the two interactions is shown in Table 1. We also tested binding of the FGFR2 fragment to the FGL peptide (Fig. 2C), previously shown to bind FGFR1 [6]. The FGL peptide corresponds to a binding site for FGFR1 in NCAM (the FG loop region of the NCAM F3 module 2). The calculated K_d value for the FGFR2-FGL interaction was very similar to that for the FGFR1-FGL interaction (see Table 1).

Thus, it appears from these experiments, that NCAM can bind FGFR2, and that the NCAM sites for FGFR1 and FGFR2 may be similar.

2.2. Analysis of the effect of ATP on the NCAM–FGFR2 interaction

Previously it has been shown that the NCAM–FGFR1 interaction can be inhibited by ATP [6]. ATP was shown to inhibit the NCAM–FGFR1 interaction by means of a competitive inhibition mechanism, namely: ATP bound to the ATP binding motif located in the FG loop region of the F3 module 2 of NCAM, and this region of NCAM was also shown to be a binding site for FGFR1 [6]. Since the NCAM sites for FGFR1 and FGFR2 appear to be similar, the NCAM–FGFR2 interaction could also be expected to be inhibited by ATP. To test this assumption, binding of the F3 modules 1–2 of NCAM and the FGL peptide to the FGFR2 Ig modules 2–3 in the presence of the various concentrations of ATP was studied by SPR. As appears from Fig. 3A and B, ATP inhibited binding to the FGFR2 fragment of both the F3 modules 1–2 of NCAM and the FGL peptide. In order to compare the effect of ATP on the NCAM–FGFR2 interaction with that of the NCAM–FGFR1 interaction, the inhibition constant (K_i) should be calculated. However, due to the fact that the highest concentration of ATP (5 mM) did not inhibit the binding completely, and because it was not possible to measure precisely the initial binding rate due to very fast association, it was not possible to calculate the K_i value employing the same methodology as the one used for the NCAM–FGFR1 interaction. As a rough estimate of the K_i value, the ATP concentration that inhibited binding by 50% (IC50) was therefore determined. The IC50 value for the NCAM–FGFR2 interaction was estimated as approximately 5.2 mM (see Table 1), which is 14-fold higher than the 0.37 mM K_i value found for the NCAM–FGFR1 interaction. Thus, it appears from these

Table 1
Summary of the kinetic parameters for the interaction between Ig module 2–3 of FGFR1/FGFR2 and FGF1 or NCAM F3 modules 1–2 or FGL peptide

	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_d (M)	K_i/IC50 (M)
FGFR2/FGF1	$3.85 \pm 1.11 \times 10^5$	$1.44 \pm 0.14 \times 10^{-2}$	$4.91 \pm 1.56 \times 10^{-8}$	
FGFR1/FGF1	$3.14 \pm 0.62 \times 10^5$	$1.65 \pm 0.10 \times 10^{-2}$	$5.60 \pm 0.93 \times 10^{-8}$	
FGFR2/NCAM	$8.10 \pm 2.81 \times 10^3$	$7.42 \pm 0.10 \times 10^{-2}$	$9.21 \pm 3.14 \times 10^{-6}$	$5.22 \pm 2.09 \times 10^{-3}$ (IC50)
FGFR1/NCAM[a]	$8.89 \pm 3.32 \times 10^2$	$5.33 \pm 0.07 \times 10^{-3}$	$9.97 \pm 0.37 \times 10^{-6}$	$3.70 \pm 0.01 \times 10^{-4}$ (K_i)
FGFR2/FGL	$5.31 \pm 2.43 \times 10^3$	$1.01 \pm 0.01 \times 10^{-2}$	$1.75 \pm 1.39 \times 10^{-6}$	$8.00 \pm 3.79 \times 10^{-3}$ (IC50)
FGFR1/FGL[a]			$2.58 \pm 2.06 \times 10^{-6}$	

[a]Data from Kiselyov et al. [6].

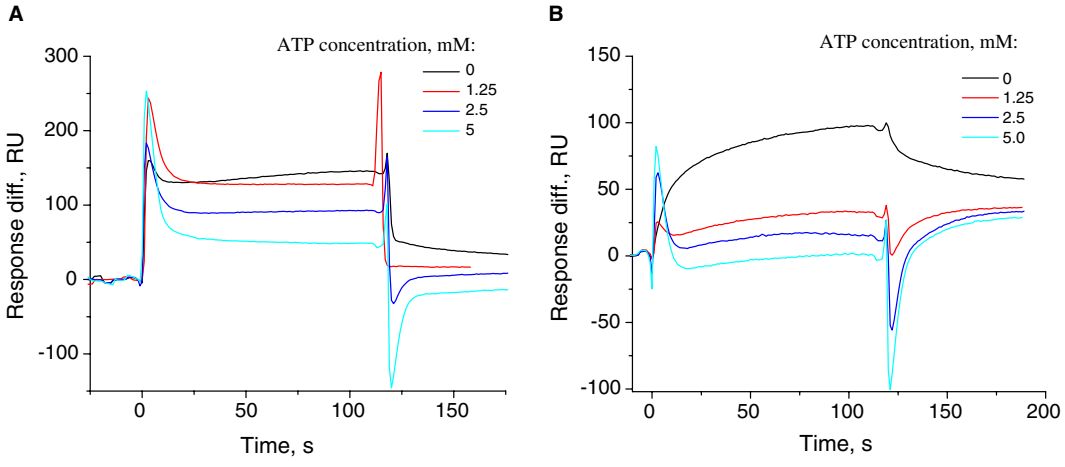

Fig. 3. Demonstration of an inhibition by ATP of the binding between the FGFR2 Ig modules 2–3 and the NCAM F3 modules 1–2 or the FGL peptide. (A) Binding of the 35 μM NCAM fragment, and (B) binding of the 5 μM FGL peptide in the presence of ATP at the specified concentrations.

experiments that ATP can inhibit the NCAM–FGFR2 interaction. However, ATP seems to be 14 times less effective in inhibiting the NCAM–FGFR2 binding in comparison to the NCAM–FGFR1 binding.

3. Discussion

In this study we have shown that the NCAM F3 modules 1–2 bound to the FGFR2 Ig modules 2–3 with a K_d value of 9.21 μM, which is very similar to the 10 μM K_d value for the NCAM–FGFR1 interaction. Since the NCAM concentration in the membrane of neurons was estimated to be on average 50 μM, then according to previous calculations [6], around 85% of the FGFR2 molecules are expected to be bound by NCAM under physiological conditions. For the NCAM–FGFR1 interaction, approximately 83% of the FGFR1 molecules are expected to be bound by NCAM under the same conditions. Based on the fact that the NCAM–FGFR1 interaction can lead to activation of FGFR1, and the fact that the calculated percentages of FGFR1 and FGFR2 molecules expected to be bound by NCAM are similar, we propose that NCAM also can activate FGFR2.

Both the NCAM–FGFR1 and NCAM–FGFR2 interactions are characterized by relatively fast association and dissociation phases. From the kinetic parameters of these interactions (see Table 1), we can estimate that the stability of the NCAM–FGFR1 complex is approximately 200 s, and that of the NCAM–FGFR2 – approximately 15 s. Thus, FGFR1 and FGFR2 cycle between the NCAM bound and unbound forms relatively quickly, but the FGFR2 cycling is approximately 13 times faster than that of FGFR1. The possible physiological significance of the faster cycling by FGFR2 is not immediately apparent, because compared to the time scale of a physiological response such as neurite-induction, which requires many hours (possibly days), the stability of both complexes is negligible. However, NCAM may be expected to be less effective in activating FGFR2 than FGFR1, if the time-period required for activation of FGFR is longer than the stability of the NCAM–FGFR2 complex. Whether or not this is true requires further investigation.

The FGL peptide bound to FGFR2 with an affinity similar to that of the FGL–FGFR1 interaction. This indicates that the NCAM sites binding to FGFR2 and FGFR1 are similar, namely: the FG loop region of the NCAM F3 module 2. This is further supported by the fact that ATP, which binds the ATP binding motif located in the NCAM F3 module 2, inhibited the NCAM–FGFR2 interaction. However, ATP was found to be 14 times less effective in inhibiting the NCAM–FGFR2 interaction compared to the NCAM–FGFR1 interaction, indicating that although the NCAM sites binding to FGFR1 and FGFR2 are similar, they are not identical. Taking into consideration that the highest local concentration of ATP in synaptic vesicles is approximately 1 mM, if in free solution [2], and the fact that ATP inhibits the NCAM–FGFR2 binding with an IC50 value of approximately 5 mM, ATP is not expected to significantly affect the NCAM–FGFR2 binding under physiological conditions, and thus play a role in regulation of the NCAM–FGFR2 interaction. However, a regulatory role of ATP cannot be entirely excluded.

Thus, in this study we demonstrated that the structural parameters and affinity of the NCAM–FGFR2 binding are similar to that of the NCAM–FGFR1 binding, which allows us to presume that NCAM can activate FGFR2 in the same way as it activates FGFR1.

4. Methods

4.1. Production of recombinant proteins

The combined Ig2–3 modules of rat FGFR2 consist of a His-tag, AGHHHHHHE, and amino acids 166–384 (swissprot Q63237). The FGFR2 construct was expressed in the KM71 strain of yeast *P. pastoris* (Invitrogen, USA) according to the manufacturer's instructions. The NCAM F3 modules 1–2 were produced in *P. pastoris* as previously described [6]. The FGFR2 and NCAM constructs were purified by affinity chromatography using Ni^{2+}-NTA resin (Qiagen, USA) and/or ion exchange chromatography and gel filtration. The rat full-length FGF1 (amino acids 1–155, swissprot P61149) was expressed in a TOP10F′ strain of *Escherichia coli* (Invitrogen) and purified by affinity chromatography using heparin resin.

4.2. SPR analysis

Binding analysis was performed using a BIAcoreX instrument (Biosensor AB, Sweden) at 25 °C using 10 mM sodium phosphate, pH 7.4, 150 mM NaCl as running buffer. The flow-rate was 5 μl/min. The Ig2–3 modules of FGFR1 or FGFR2 were immobilized on the sensor chip CM5 (Biosensor AB, Sweden) as previously described [6]. Approx. 2000 resonance units (RU) of the FGFR modules were immobilized on the sensor chip. Binding was studied in the following way: A compound was injected at a specified concentration simultaneously into a flow-cell with the immobilized FGFR modules (Fc1-cell) and a control flow-cell with nothing immobilized (Fc2-cell). The curve representing unspecific binding of the compound to the surface of the Fc2-cell was subtracted from the curve representing binding of the same compound to the immobilized Ig2–3 modules and the surface of the Fc1-cell. The resulting curve was used for analysis. The kinetic constants were calculated from the dissociation and association phases using the manufacture's software. Three independent experiments were performed.

Acknowledgments: This work was supported by grants from Købmand i Odense Johann og Hanne Weimann, f. Seedorffs Legat (to V.V.K.), The Danish Medical Research Council (to E.B. and V.B.), The Danish Cancer Society (to E.B. and C.C.), and the Lundbeck Foundation (to E.B. and V.B.), and the EU integrated project PROMEMORIA.

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