

Nerve Growth Factor Signals via Preexisting TrkA Receptor Oligomers

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ABSTRACT Nerve growth factor (NGF) promotes neuronal survival and differentiation by activating TrkA receptors. Similar to other receptor tyrosine kinases, ligand-induced dimerization is thought to be required for TrkA receptor activation. To study this process, we expressed TrkA receptors in *Xenopus laevis* oocytes and analyzed their response to NGF by using a combination of functional, biochemical, and structural approaches. TrkA receptor protein was detected in the membrane fraction of oocytes injected with TrkA receptor cRNA, but not in uninjected or mock-injected oocytes. Application of NGF to TrkA receptor-expressing oocytes promoted tyrosine phosphorylation and activated an oscillating transmembrane inward current, indicating that the TrkA receptors were functional. Freeze-fracture electron microscopic analysis demonstrated novel transmembrane particles in the P-face (protoplasmic face) of oocytes injected with TrkA cRNA, but not in uninjected or mock injected oocytes. Incubating TrkA cRNA-injected oocytes with the transcriptional inhibitor actinomycin D did not prevent the appearance of these P-face particles or electrophysiological responses to NGF, demonstrating that they did not arise from de novo transcription of an endogenous *Xenopus* oocyte gene. The appearance of these particles in the plasma membrane correlated with responsiveness to NGF as detected by electrophysiological analysis and receptor phosphorylation, indicating that these novel P-face particles were TrkA receptors. The dimensions of these particles (8.6×10 nm) were too large to be accounted for by TrkA monomers, suggesting the formation of TrkA receptor oligomers. Application of NGF did not lead to a discernible change in the size or shape of these TrkA receptor particles during an active response. These results indicate that in *Xenopus* oocytes, NGF activates signaling via pre-formed TrkA receptor oligomers.

INTRODUCTION

Nerve growth factor (NGF) is required for the development and maintenance of the nervous system. NGF initiates many of its biological effects by activating TrkA tyrosine kinase receptors in the plasma membrane (Barbacid, 1995; Klesse and Parada, 1999; Kaplan and Miller, 2000). Similar to other receptor tyrosine kinases, NGF is thought to induce receptor dimerization as a first step for signal transduction (Meakin and Shooter, 1991; Schlessinger and Ullrich, 1992). This hypothesis is supported by the bivalent nature of NGF that can bring TrkA receptor monomers into close proximity such that each monomer phosphorylates multiple tyrosine residues on its partner, thereby initiating downstream signal transduction (Kaplan and Stephens, 1994; McDonald et al., 1991; Meakin and Shooter, 1991; Schlessinger and Ullrich, 1992). However, several studies have shown that biological response can be elicited with monovalent ligands or a chimeric NGF/neurotrophin-4 dimer (Maliartchouk et al., 2000a,b; Treanor et al., 1995). Therefore, ligand binding per se may not be responsible for receptor dimerization. In line with these observations, recent studies of other receptor tyrosine kinases suggest that

1) tyrosine kinase receptors can form ligand-independent oligomers on the plasma membrane of cells (Gadella and Jovin, 1995; Wiseman and Petersen, 1999); 2) dimerization/oligomerization of these receptor tyrosine kinases is necessary but not sufficient to activate signaling; and 3) binding of ligand induces rotational coupling of these pre-formed receptor oligomers to initiate receptor transphosphorylation and signal transduction (Jiang and Hunter, 1999; Burke et al., 1997; Burke and Stern, 1998; Livnah et al., 1996, 1998, 1999; Remy et al., 1999).

To investigate the oligomeric state (i.e., monomer, dimer, or larger size oligomer) of TrkA receptors and the possible role of NGF binding in inducing oligomerization, we expressed TrkA receptors in *Xenopus laevis* oocytes and examined their response to NGF by using a combination of functional, biochemical, and structural approaches. Two properties made oocytes ideally suited for structure/function studies. First, the plasma membrane of the oocyte contains a low density of endogenous transmembrane proteins ($200\text{--}300/\mu\text{m}^2$ instead of $>2000/\mu\text{m}^2$ in most cells), which allows the identification of the exogenous proteins from the size and shape of the newly inserted particles induced by cRNA injection. Second, oocytes synthesize and transport to the plasma membrane a large number of exogenous channels and transporters as well as many of their genetically engineered mutants. While in the plasma membrane, the exogenous proteins are characterized by electrophysiological and optical methods before the same oocytes are processed for electron microscopy (Eskandari et al., 1998, 1999, 2000; Zampighi et al., 1995, 1999). These properties of the oocyte expression system provide a simple yet pow-

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erful approach to determine whether functional membrane proteins are assembled as monomers, dimers, or larger size oligomers in a heterologous cellular environment.

MATERIALS AND METHODS

cRNA preparation and oocyte microinjection

The carboxy-terminal FLAG-tagged TrkA receptor was generated by a PCR strategy to insert two FLAG epitopes in tandem on the carboxy-terminal (Mischel et al., 2001). A PCR reaction was done with the rat TrkA cDNA as a template, using forward primer 5'CA GGG ACT AGT GGT CAA GAT GAT TGG A and reverse primer 5' CTT ATC ATC ATC ATC CTT GTA ATC GCC CAG AAC GTC CAG G to amplify a fragment encoding the last 405 basepairs of rat TrkA receptor coding sequence (minus the stop codon) including the 5'*Spe*I site (part of the coding sequence) and the first FLAG epitope on the 3' end. This product was then used as a template for a second PCR reaction using the same forward primer and a reverse primer encoding the second FLAG tag followed by a stop codon and *Xho*I restriction site 5'CTG CTC GAG CTA CTT ATC ATC ATC ATC CTT GTA ATC CTT ATC ATC ATC. The amplified product was digested with *Spe*I and *Xho*I and subcloned into similarly digested full-length TrkA in pGEMHE (a modified pGEM vector that contains 5' and 3' *Xenopus* β -globin untranslated sequences for RNA stability (Liman et al., 1992; Mischel et al., 2001). TrkA was transcribed using the T7 mMessage Machine from Ambion (Austin, TX). Transcripts were purified using an RNaeasy kit purchased from QIAGEN Inc. (Valencia, CA), analyzed on agarose gels, and quantitated by spectrophotometry (and by densitometric comparison to RNA standards).

Mature *Xenopus laevis* oocytes (Dumont stage V–VI) were isolated, defolliculated, and injected with 5 ng of TrkA cRNA. Oocytes were maintained at 17–18°C in ND96 (in mM): 96 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 HEPES, pH 7.5, and 1 μ g/ml ciprofloxacin, 6 μ g/ml ceftazidime, and 1 μ g/ml gentamicin.

Immunoblot analysis

For analysis of TrkA expression, 10 oocytes microinjected with TrkA cRNA (and 10 control oocytes) were manually homogenized in an ice-cold solubilization buffer (65 mM Tris-HCl, 50 mM NaCl, 5 mM EDTA, 2% Triton X-100, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 5 μ g/ml aprotinin, 5 μ g/ml leupeptin, 1 mM sodium orthovanadate, 10 mM sodium fluoride, pH 7.5). Lysates were incubated at 4°C for 1 h and the soluble phase was partitioned from the insoluble and lipid phases by three rounds of centrifugation at 18370 \times g for 5 min at 4°C. For preparation of a membrane fraction, oocytes were manually homogenized in ice-cold homogenization buffer and samples were centrifuged at 735 \times g for 5 min at 4°C. The pellet (containing the membrane) was solubilized in 500 μ l of a buffer containing 130 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2% Triton X-100 and 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, and 1 mM sodium orthovanadate. Samples were pre-cleared with protein A/G Sepharose beads (Pierce, Rockford, IL), and immunoprecipitated with anti-FLAG M2 antibody (Stratagene, La Jolla, CA). Immunoprecipitates were washed three times with 1 ml of solubilization buffer and protein was recovered by boiling for 5 min in 3 \times Laemmli sample buffer. SDS-PAGE was performed on 7.5% polyacrylamide gels. After transfer to nitrocellulose membranes (Hybond, Amersham Pharmacia Biotech, Piscataway, NJ), samples were blocked in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 8.0) with 5% non-fat dried milk and incubated with the anti-FLAG M2 monoclonal antibody (Stratagene) at a concentration of 10 μ g/ml overnight at 4°C. After washing, blots were incubated with horseradish peroxidase (HRP) conjugated goat anti-mouse antibody (Promega, Madison, WI) or HRP conjugated goat anti-rabbit antibody (Promega) at a

dilution of 1:5000 for 1–2 h at room temperature. After washing, bands were visualized by incubating with Supersignal (Pierce), followed by exposure to Kodak BIOMAX film (Eastman Kodak Company, Rochester, NY). Experiments were repeated three times using independent oocytes.

For analysis of tyrosine phosphorylation, 100 ng·ml⁻¹ of NGF (4 nM) (Sigma, St. Louis, MO) was added to the media of 10 TrkA-expressing oocytes and 10 control oocytes for 5 min at room temperature. Oocytes were manually homogenized in ice-cold solubilization buffer, incubated for 1 h at 4°C, and centrifuged at 18370 \times g for 5 min at 4°C. Samples were resolved electrophoretically for immunoblot analysis as mentioned above, and incubated with anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology, Lake Placid, NY). Blots were washed as above and incubated with HRP conjugated goat anti-mouse antibody (Promega), and visualized by using enhanced chemiluminescence (Amersham Pharmacia Biotech). Experiments were repeated three times using independent oocytes.

For analysis of endogenous NGF expression in oocytes, 20 uninjected oocytes were incubated in 60 μ l of ND96 for 24 h. Oocytes were manually homogenized in ice-cold solubilization buffer, solubilized and fractionated as described above. Samples were separated electrophoretically for immunoblot analysis and incubated with anti-2.5S-NGF monoclonal antibody (Sigma). For analysis of NGF expression in the oocyte culture medium, 20 μ l (one-third of total culture medium) was resolved electrophoretically for immunoblot analysis.

Electrophysiology

Stage V–VI *Xenopus laevis* oocytes were isolated, defolliculated, injected with TrkA cRNA (5–20 ng), and cultured for 2 days in Barth's solution (Umbach et al., 1990). Oocytes with resting potentials more negative than -50 mV were voltage-clamped using the two-electrode voltage clamp method and an Axoclamp-2A amplifier (Axon Instruments, Foster City, CA). The membrane potential was held at -60 mV and current measurements were made at 20–22°C during bath application of NGF (100 ng·ml⁻¹) in Barth's solution. Data were recorded on a PrimeLine (Soltec, San Fernando, CA) chart recorder and also stored on Axotape (Axon Instruments) for analysis. The reversal potential of the NGF-evoked current was estimated by stepping the holding potential to depolarizing potentials during an active response (Umbach et al., 1990). Membrane capacitance was determined at the beginning of each experiment by integrating (using Clampfit, pCLAMP 6.0, Axon Instruments) the capacitive current elicited by +10 and +20 mV depolarizing steps (30 ms) from the holding membrane potential (Zampighi et al., 1999). Whole-cell capacitance is reported as the mean calculated from at least six pulses per cell. Immediately following electrophysiological measurements, oocytes were fixed in 4% glutaraldehyde in 0.2 M sodium cacodylate (pH 7.4) (Zampighi et al., 1999), and prepared for freeze-fracture electron microscopy (Eskandari et al., 1998, 1999, 2000; Zampighi et al., 1999).

Freeze-fracture electron microscopy

The freeze-fracture structural data were collected from a total of eight oocytes (3 TrkA-expressing oocytes treated with NGF, 3 TrkA-expressing oocytes without NGF treatment, and 2 control uninjected oocytes). For particle density determinations, images of protoplasmic (P) fracture faces were digitized at a final magnification of 150,000 \times . Particle densities were determined by counting P-face particles from known areas of the plasma membrane (NIH Image). For each oocyte, a minimum of 1000 particles was counted from at least 10 μ m² of plasma membrane. For measurement of the dimensions of the freeze-fracture particles, particles were sampled from two replicas from two oocytes. For construction of the frequency histograms, the particle diameter was measured directly from the negatives using a comparator (Nikon, Model 6c) at a final magnification of 1,000,000 \times . The diameter measurements were plotted in a frequency

histogram with a bin size of 1.0 nm and were fitted to a multiple Gaussian function (Eskandari et al., 1998, 1999, 2000; Zampighi et al., 1999) (see Fig. 3). All values are reported as mean \pm SE.

RESULTS

Expression of functional TrkA receptors in *Xenopus* oocytes

As a prelude to the morphometric characterization of TrkA receptors expressed in the plasma membrane of *Xenopus* oocytes, we performed experiments to verify that functional TrkA receptors were expressed in these cells. Prior investigations have documented, and we have confirmed (data not shown), that *Xenopus* oocytes do not express either TrkA or p75NTR receptors (Mischel et al., 2001; Nebreda et al., 1991; Sehgal et al., 1988). Injection of *Xenopus* oocytes with TrkA receptor cRNA led to the appearance of a 140/110 kDa TrkA immunoreactive doublet, which was not detected in uninjected (Fig. 1 A) or water injected oocytes (data not shown) (Carriero et al., 1991; Mischel et al., 2001; Nebreda et al., 1991). The 140 kDa band, which represents the fully glycosylated membrane-bound receptor (Barbacid, 1995; Meakin and Shooter, 1991) was also present in a plasma membrane fraction isolated from TrkA receptor cRNA-injected oocytes, but not in control oocytes (Fig. 1 A) (Mischel et al., 2001). These TrkA receptors were catalytically active, as demonstrated by tyrosine phosphorylation of a number of proteins, including TrkA receptors, in response to NGF (Fig. 1 B).

Further evidence that the 140/110 kDa immunoreactive species of Fig. 1, A and B corresponded to functional TrkA receptors came from the electrophysiological analysis of oocytes expressing TrkA. In the absence of NGF the resting electrophysiological properties of TrkA-expressing oocytes did not differ from those of uninjected or water-injected oocytes (not shown). Bath application of a saturating concentration of NGF ($100 \text{ ng}\cdot\text{ml}^{-1}$) elicited a slowly developing (10–15 s) oscillating inward current in oocytes injected with TrkA receptor cRNA (Fig. 1 C). This NGF-evoked current was not seen in control oocytes, even after several minutes of exposure to this ligand (Fig. 1 C). Overall, 10 oocytes injected with TrkA receptor cRNA exhibited responses similar to that seen in Fig. 1 C, while uninjected oocytes ($n = 5$) were uniformly unresponsive to NGF.

Freeze-fracture analysis of the plasma membrane of oocytes

To investigate whether NGF induces oligomerization of TrkA receptors, we used freeze-fracture electron microscopy to examine TrkA receptors expressed in the plasma membrane of oocytes. First, we characterized the plasma membrane particles of control oocytes. The P-face of control cells contained a low density of particles at 200 ± 10

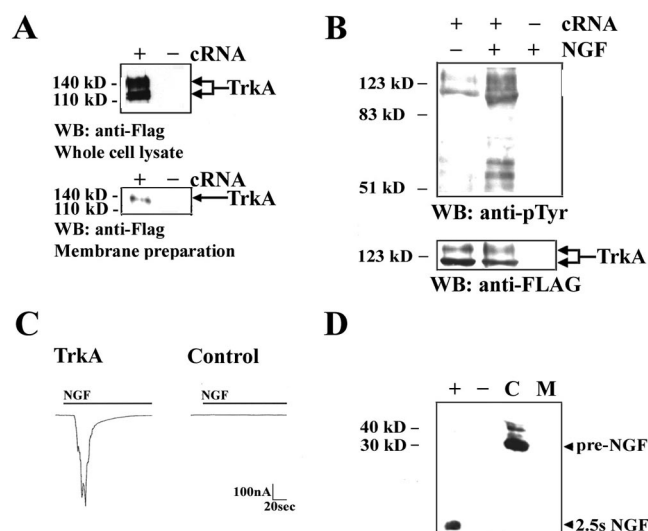
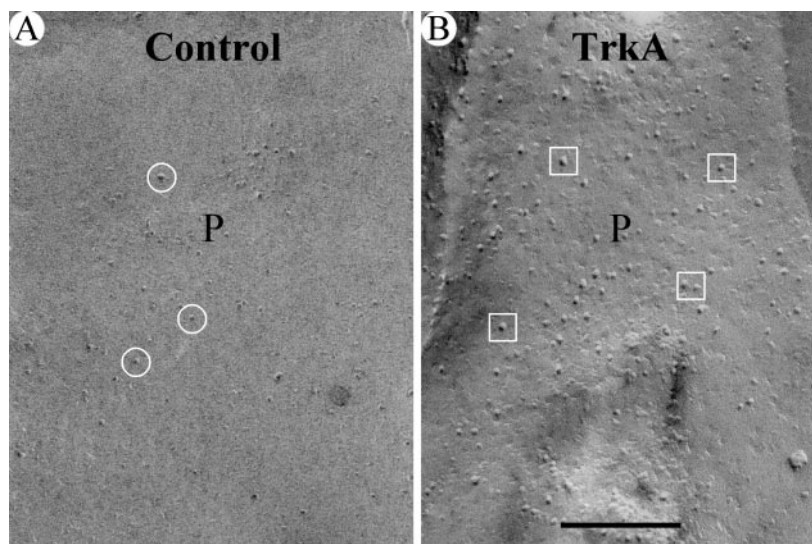


FIGURE 1 Functional expression of TrkA receptors in the plasma membrane of *Xenopus* oocytes. (A) Immunoblot analysis. Whole-cell lysates (top panel) or membrane fractions (bottom panel) from oocytes microinjected with FLAG-tagged TrkA receptor cRNA (lane 1) or control oocytes (lane 2) immunoprecipitated with an antibody against the FLAG epitope and immunoblotted with the same antibody. (B) NGF stimulates tyrosine phosphorylation of expressed TrkA receptors. Whole-cell lysates from oocytes expressing TrkA, or control oocytes that were stimulated with NGF ($100 \text{ ng}\cdot\text{ml}^{-1}$) for 5 min were immunoblotted with anti-phosphotyrosine antibody (top panel). Lysates probed with anti-FLAG antibody (bottom panel) demonstrate equal amounts of TrkA receptor protein in oocytes with or without addition of NGF. The position of TrkA receptors is marked with arrows. (C) In TrkA-expressing oocytes, NGF ($100 \text{ ng}\cdot\text{ml}^{-1}$) activated a transient inward current with a notable oscillatory behavior. The reversal potential for the NGF-evoked current was -29 mV (not shown). In control oocytes there was no response to NGF. (D) Oocytes do not secrete endogenous NGF. Lysates from cells (C) or one-third of the total conditioned media (M) from control oocytes immunoblotted with anti-NGF antibody. Immature NGF precursor (32 kDa, right arrowhead) is seen in cells but not in the oocyte-bathing medium. No mature NGF is seen in the cytoplasm or in the bathing medium of the oocytes. The anti-NGF antibody recognizes the 2.5 s NGF (+) control ($1 \mu\text{g}$ of NGF; right arrow).

per μm^2 (Fig. 2 A). Consistent with previous observations (Eskandari et al., 1998, 1999, 2000; Zampighi et al., 1999), these endogenous P-face particles were circular in cross-section with a mean diameter of $7.6 \pm 0.5 \text{ nm}$ (Fig. 2 A). Independent assessment of exoplasmic (E) face particles revealed a density of 890 ± 40 per μm^2 (Eskandari et al., 1999). Water-injected oocytes exhibit a similar population of P-face and E-face particles (data not shown). We then examined the freeze-fracture particles of oocytes expressing TrkA in the absence of exposure to NGF. Oocytes expressing the TrkA receptor revealed an additional population of large particles in the P face of the plasma membrane (Fig. 2 B and Fig. 3, A and C). High magnification analysis ($1,000,000\times$) of a selected subset of these particles ($n = 48$) demonstrated a rectangular shape with axes measuring $10 \pm 1.3 \text{ nm} \times 8.6 \pm 0.5 \text{ nm}$ (Figs. 2 B and 3); $a/b = 1.16 \pm 0.05$. These particles were randomly oriented in the xy -

FIGURE 2 Freeze-fracture images of the P-face of the plasma membrane of a control oocyte and an oocyte expressing TrkA. In freeze-fracture images integral membrane proteins appear as “particles.” In the control cell (A) the density of the P-face particles was $200 \pm 10/\mu\text{m}^2$ ($n = 6$ fracture faces). In the oocyte expressing TrkA (B), the density increased to $340 \pm 40/\mu\text{m}^2$ ($n = 11$ fracture faces). Using the density and the total surface area of the same cell (obtained from whole-cell capacitance), it is estimated that there were 3.4×10^9 TrkA receptors in the plasma membrane of the cell in (B). Several endogenous particles in (A) are enclosed in circles, and those arising from TrkA expression (B) are enclosed in squares. P denotes the protoplasmic face of the plasma membrane. Scale bar for A and B, 160 nm.



plane. The presence of this new population of particles in the plasma membrane led to an increase in the total density of P-face particles to $340 \text{ particles}/\mu\text{m}^2$, an increase of $\sim 140 \text{ particles}/\mu\text{m}^2$. No change in the density of E-face particles was observed. This partitioning of the TrkA receptors to the P-face of the membrane is consistent with other heterologously expressed integral membrane proteins (Eskandari et al., 1998, 1999, 2000; Zampighi et al., 1995, 1999). To exclude the possibility that these particles resulted from the expression of an endogenous membrane protein initiated by the injection of TrkA receptor cRNA, we cultured the oocytes in actinomycin D ($0.1 \text{ mg}\cdot\text{ml}^{-1}$), which inhibits transcription of endogenous genes (Umbach and Gunderson, 1987). This treatment did not block the appearance of the novel TrkA-induced particles, or electrophysiological responses of oocytes to NGF (data not shown). Therefore, the $10 \times 8.6 \text{ nm}$ freeze-fracture particles that appeared after injection of TrkA cRNA arose specifically from translation of this foreign cRNA. Using whole-cell capacitance measured in the same cell as an index of the cell surface area (Zampighi et al., 1999), we estimated that there were $\approx 3.4 \times 10^9$ TrkA receptor particles present in the plasma membrane of the oocytes analyzed.

Xenopus oocytes do not secrete NGF

NGF mRNA is present in *Xenopus* oocytes, and an immature NGF precursor protein has been observed in the cytoplasm of oocytes (Carriero et al., 1991). However, mature NGF protein has not been detected in oocytes or their secreted medium (Carriero et al., 1991; Wion et al., 1984). Similarly, neurotrophic activity could not be detected in the conditioned medium of oocytes or ovarian follicles (Sehgal et al., 1988). We detected a 32 kDa immunoreactive band in the cytoplasmic fraction of oocytes (Fig. 1 D) consistent with the NGF precursor, as has previously been demon-

strated (Carriero et al., 1991; Wion et al., 1984). However, the mature form of NGF was not detected in the cytoplasm or culture medium of oocytes (Fig. 1 D). Similarly, no mature NGF was detected in the culture medium from water-injected or TrkA cRNA-injected oocytes (data not shown). These findings argue that oocytes do not secrete NGF, consistent with our electrophysiological result in which the large NGF-evoked inward currents (Fig. 1 C) were observed only after addition of NGF to the oocyte bathing medium. For NGF to have provoked the responses exemplified in Fig. 1 C, there must have been unliganded TrkA receptors in the plasma membrane. Therefore, these results suggest that the $10 \times 8.6 \text{ nm}$ TrkA receptor particles in the plasma membrane (Figs. 2 and 3 A) correspond to the inactive form of the receptor before NGF application.

NGF does not induce TrkA receptor aggregation

To determine whether NGF induces TrkA receptor aggregation, we studied the size and shape of freeze-fracture particles in oocytes fixed during an active response to the ligand, as determined by the inward transmembrane current (as illustrated by the response in Fig. 1 C). Simple visual inspection showed that dimensions of the P and E freeze-fracture particles remained unchanged upon addition of NGF. This qualitative assessment was confirmed by comparing size frequency histograms of P-face particles in the plasma membrane of NGF-treated and untreated oocytes (Fig. 3, C and D). For the ligand-induced dimerization hypothesis to be correct, the $10 \times 8.6 \text{ nm}$ TrkA receptor particles would have to form substantially larger ($20 \times 8.6 \text{ nm}$ or $10 \times 17.2 \text{ nm}$) freeze-fracture particles. It can be argued that the electrical response elicited from saturating concentration of NGF might have resulted in the dimerization of just a few receptor molecules. However, the predicted $20 \times 8.6 \text{ nm}$ or $10 \times 17.2 \text{ nm}$ diameter particles are

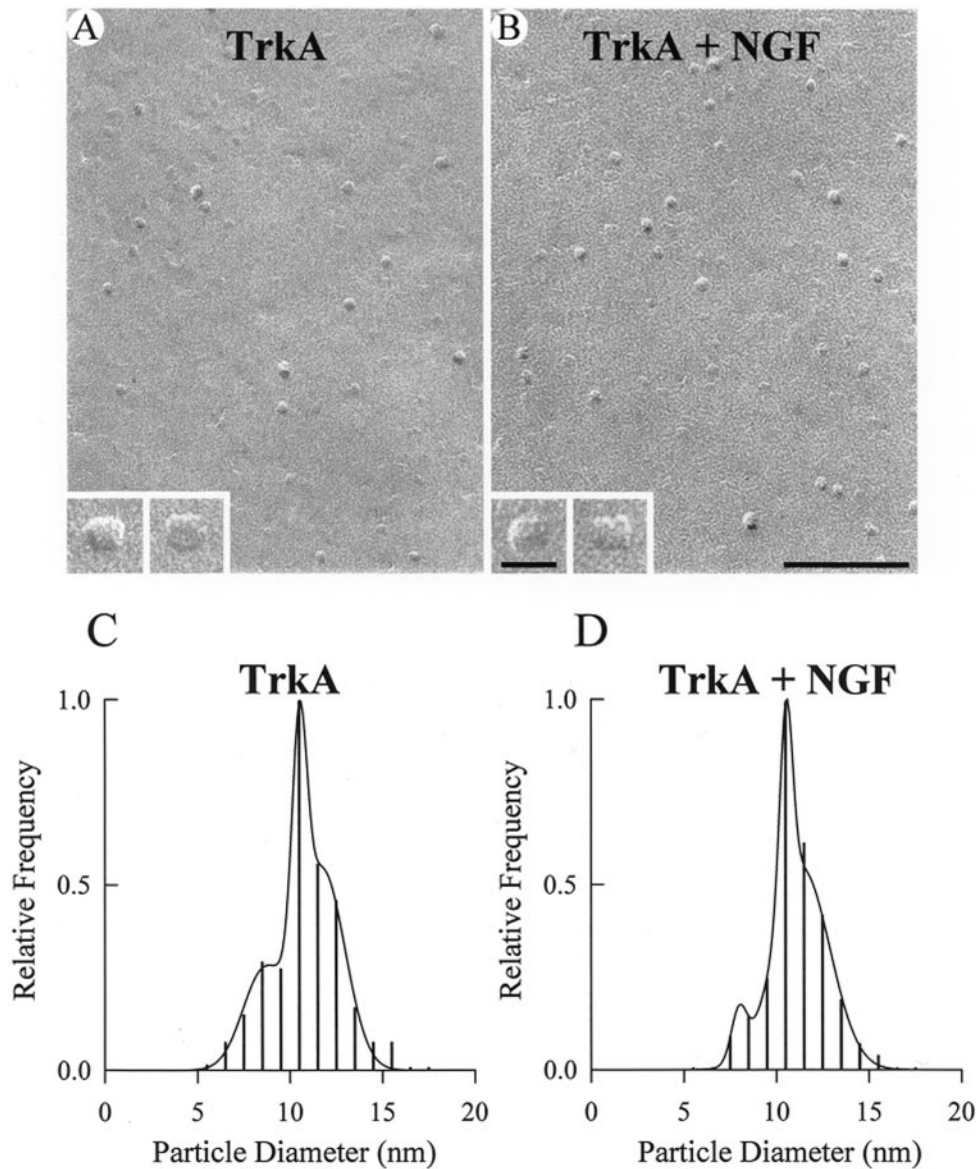


FIGURE 3 P-face of the plasma membrane of TrkA-expressing oocytes with or without exposure to NGF. The density of P-face particles is similar in the TrkA-expressing oocytes regardless of exposure to NGF. In the absence of NGF (A) the particle density was $340 \pm 40/\mu\text{m}^2$ ($n = 11$ fracture faces), and in the presence of NGF (B) the density was $340 \pm 90/\mu\text{m}^2$ ($n = 9$ fracture faces). Most importantly, no change occurred in the size and/or shape of the TrkA particles after incubation of the cells with NGF (insets in A and B). (C) Size distribution of P-face particles in TrkA-expressing oocytes not exposed to NGF. In addition to the population of endogenous particles, there was a predominant exogenous population that appeared only after expression of TrkA (140 ± 90 particles per μm^2). (D) The size distribution of the particles induced by TrkA expression was not altered if the cell was incubated in NGF before preparation for freeze-fracture microscopy. The dimensions of TrkA receptor particles were the same in the absence and presence ($100 \text{ ng}\cdot\text{ml}^{-1}$) of NGF ($10 \pm 1.3 \text{ nm} \times 8.6 \pm 0.5 \text{ nm}$). Because the density of endogenous particles varies considerably between individual oocytes ($150\text{--}350/\mu\text{m}^2$) (11–15), the difference in the relative frequency of endogenous particles in the TrkA injected oocytes with and without NGF is not significant. The histograms in (C) and (D) were generated from a total of 8497 particles counted, 4625 particles in untreated TrkA-expressing oocytes, and 3862 particles in NGF-treated, TrkA-expressing oocytes ($250,000\text{--}500,000\times$). High-magnification analysis ($1,000,000\times$), which demonstrated the rectangular shape of the particles, was performed on a selected subset ($n = 48$). Scale bar for (A) and (B), 80 nm. Scale bar for the insets in (A) and (B), 15 nm.

so large with respect to the size distribution observed in untreated oocytes that it would have been easily detected by simple visual inspection of the large number of replicas examined. Therefore, NGF does not alter the dimensions of the TrkA receptor particles in the plasma membrane of the oocyte during an active response.

DISCUSSION

It has been proposed that ligand-induced dimerization of TrkA receptor monomers is a prerequisite for NGF signaling (Jing et al., 1992; Meakin and Shooter, 1991; Schlessinger and Ullrich, 1992). NGF, which is bivalent (McDonald

et al., 1991), is thought to bridge two TrkA receptor monomers, allowing each monomer to phosphorylate its partner. The finding that NGF promotes chemical cross-linking of TrkA receptors lends support to this hypothesis (Jing et al., 1992; Meakin and Shooter, 1991; Schlessinger and Ullrich, 1992). However, evidence obtained from the use of monovalent ligands that cannot bridge TrkA receptors suggests that ligand binding per se may not be responsible for receptor dimerization (Maliartchouk et al., 2000a; Treanor et al., 1995). To test this hypothesis, we combined electrophysiological, biochemical, and freeze-fracture electron microscopy data to study the effect of NGF treatment on the structure and function of TrkA receptors expressed in the plasma membrane of oocytes.

Our data indicate that functional TrkA receptors were expressed in the plasma membrane of oocytes injected with TrkA cRNA. Injection of TrkA cRNA correlated with the appearance of a 140/110 kDa doublet on immunoblot analysis. These TrkA receptors were catalytically active, as the TrkA receptor protein exhibited an NGF-dependent increase in tyrosine phosphorylation (Clary et al., 1994; Jing et al., 1992; Kaplan and Stephens, 1994; Kaplan and Miller, 2000; Segal and Greenberg, 1996). Electrophysiological analysis indicated that addition of NGF to the bathing medium evoked an oscillating inward current with a reversal potential of -29 mV, consistent with a chloride current. TrkA-mediated activation of phospholipase C stimulates the release of ionized calcium from cytoplasmic stores (Kaplan and Stephens, 1994; Klein et al., 1991; Loeb et al., 1994; Mischel et al., 2001; Obermeier et al., 1993; Segal and Greenberg, 1996), which activates endogenous calcium-dependent chloride channels in oocytes (Callamaras and Parker, 2000; Hartzell, 1996; Weber, 1999). The NGF-evoked current was not seen in control oocytes, indicating that the response to NGF was via the heterologously expressed TrkA receptors.

To visualize the TrkA receptors on the plasma membrane we performed freeze-fracture electron microscopy. This method splits plasma membranes to produce complementary P and E fracture faces, where integral membrane proteins appear as "particles" in the fracture faces (Branton, 1966; Eskandari et al., 1998, 1999, 2000; Zampighi et al., 1999). Proteins heterologously expressed in oocytes appear as distinct P-face particles whose size and shape can be distinguished from the endogenous integral membrane proteins (Eskandari et al., 1998, 1999, 2000; Zampighi et al., 1999). This technique can resolve proteins as small as 2.5 nm (Eskandari et al., 1999). In oocytes expressing the TrkA receptor, a new population of rectangular P-face particles (10×8.6 nm) was observed in the plasma membrane. Several lines of evidence indicate that these freeze-fracture particles represent the functional TrkA receptors in the plasma membrane observed by electrophysiological and biochemical methods. First, they were present in TrkA-expressing oocytes, but not in mock-injected or uninjected

oocytes. Second, they partitioned only within the P-face of the membrane, as has been demonstrated for a number of other heterologously expressed transmembrane proteins (Eskandari et al., 1998, 1999, 2000; Zampighi et al., 1995, 1999). Third, their presence in the plasma membrane correlated with responsiveness to NGF, as detected both by electrophysiological analysis and receptor phosphorylation. Finally, incubating oocytes with the transcriptional inhibitor actinomycin D did not prevent the appearance of the P-face particles or electrophysiological responses to NGF, indicating that they did not arise from de novo transcription of an endogenous *Xenopus* oocyte gene.

Previously, we have studied integral membrane proteins that consist of a multiple transmembrane domains connected by small loops of extracellular domain (Eskandari et al., 1998, 1999, 2000). Therefore, the cross-sectional area of these freeze-fracture particles provided an index of the number of transmembrane α helices of the proteins (1.4 ± 0.03 helix/nm³) (Eskandari et al., 1998, 1999). In contrast, TrkA receptors have a single transmembrane domain and a large globular extracellular domain (Holden et al., 1997; Wiesmann et al., 1999). Because the freeze-fracture technique results in asymmetric cleavage of the integral membrane protein, it is likely that the juxtamembrane region of the extracellular domain contributes to the area of the freeze-fracture particle. The TrkA receptor particles in the P-face of the plasma membrane measured 8.6×10 nm. After correcting for the thickness of the metal replica (2.4 nm on each side) (Eskandari et al., 1998, 1999, 2000), the TrkA receptor particles were 6.2 ± 0.5 nm \times 7.5 ± 1.3 nm. Because the crystal structure derived from the NGF binding domain of two TrkA monomers complexed to NGF is 6.0×9.5 nm (Wiesmann et al., 1999), our data suggest that each TrkA receptor particle consists of a minimum of two TrkA receptors. Alternatively, if the extracellular domain of TrkA does not contribute to the freeze-fracture particles, then each TrkA receptor particle consists of larger aggregates of TrkA receptors. In either case, the data indicate that TrkA receptors are present on the plasma membrane as pre-formed oligomers before NGF exposure.

To ensure that TrkA receptors were not oligomerized in response to endogenously secreted NGF, we examined oocytes and their culture media for evidence of NGF secretion. Uninjected oocytes and oocytes injected with mRNA isolated from submaxillary glands produce an immature 32 kDa NGF precursor protein, which is present in the cytoplasm (Carriero et al., 1991; Wion et al., 1984). However, they do not secrete detectable mature NGF (Carriero et al., 1991; Wion et al., 1984), prompting the hypothesis that oocytes lack the machinery to process it (Wion et al., 1984). We detected the 32 kDa NGF precursor protein in control and TrkA cRNA-injected oocytes, but we did not detect any NGF secretion (or more importantly, production of mature NGF). Although we cannot formally dismiss the possibility that the NGF precursor protein induces oligomerization of

TrkA receptors before its insertion in the plasma membrane, our data indicate that TrkA oligomerization is not sufficient to activate signaling. Moreover, our data show that the receptors expressed on the plasma membrane were competent to respond to NGF, suggesting that they were not occupied by NGF precursor protein.

To further investigate our hypothesis that TrkA receptors were pre-oligomerized, we proceeded to study the effect of NGF application on TrkA receptor aggregation. NGF had no discernible effect on the dimensions of the TrkA receptor particles compared to the TrkA-expressing controls, which had not been exposed to NGF. If NGF promoted aggregation of TrkA receptors to activate signaling (Jing et al., 1992; Meakin and Shooter, 1991; Schlessinger and Ullrich, 1992), we should have detected a new population of particles of approximately twice the dimensions of the unliganded receptors. The expected increase in the particle dimensions is well within the limits of resolution of the freeze-fracture replicas (≈ 2.5 nm) (Eskandari et al., 1999) and should have been evident by simple visual inspection of the replicas. Our method of Gaussian fit to study the size distribution of particles would not be able to distinguish a difference in TrkA-expressing oocytes treated with or without NGF if $<5\%$ of the TrkA receptors were responding to NGF. However, the extent of TrkA receptor phosphorylation in response to bath application of NGF (Fig. 1 *B*) strongly suggests that a sufficient number of TrkA receptors were activated that would have enabled us to detect any large particles due to NGF-mediated oligomerization. Furthermore, the predicted freeze-fracture particles (20×17.2 nm) would be so large with respect to the size distribution observed in the untreated oocytes that they would have been easily identified by visual inspection, even at particle densities below 1 per μm^2 . Therefore, our results indicate that NGF did not induce changes in the state of oligomerization of TrkA receptors in the plasma membrane.

TrkA receptors were expressed at a relatively high density in oocytes compared to PC12 cells, which are a common model of TrkA signaling (Connolly et al., 1985; Klesse and Parada, 1999). TrkA density in the oocyte plasma membrane was ~ 140 per μm^2 . If each particle represents a TrkA dimer, then there are ~ 280 TrkA receptor monomers per μm^2 in TrkA-expressing oocytes. In contrast, PC12 cells have a lower density of TrkA receptors. In PC12 cells, 2100–3000 TrkA binding sites have been estimated to be in the plasma membrane per cell (Clary et al., 1994; Hempstead et al., 1991). Because the surface area of undifferentiated PC12 cells is $\approx 290 \mu\text{m}^2$ (Connolly et al., 1985), this suggests that there are 7–10 TrkA binding sites per μm^2 . Therefore, we estimate that there is up to a 40-fold difference in the density of TrkA receptors in the plasma membrane of oocytes relative to PC12 cells. It can be argued that overexpression of TrkA receptors may contribute to their oligomerization. Although we detected a low level of phosphorylation of these receptors in the absence of NGF, TrkA

tyrosine phosphorylation was dramatically increased by NGF treatment. In addition, TrkA-expressing oocytes did not reveal a large inward current in the absence of NGF, and only exhibited this current in the presence of NGF. Therefore, our data suggest that NGF activation of constitutive TrkA receptor oligomers is physiological, and that TrkA receptor oligomerization is insufficient for TrkA signaling. This conclusion is further supported by the observation that in oocytes, oligomerization is an intrinsic property of the proteins that often occurs in the rough endoplasmic reticulum and is not a function of density of receptors in the plasma membrane (Eskandari et al., 1998, 1999, 2000; Zampighi et al., 1999). For example, the Na^+ /glucose cotransporter is monomeric over a very wide density range ($100\text{--}3500/\mu\text{m}^2$) (Eskandari et al., 1998); the neuronal glutamate transporter (EAAT3) is pentameric ($100\text{--}800/\mu\text{m}^2$) (Eskandari et al., 2000); and the gap junction hemichannel connexin50 is hexameric ($100\text{--}800/\mu\text{m}^2$) (Zampighi et al., 1999). These results suggest that oligomer formation in the absence of ligand is an intrinsic property of TrkA receptors, and is not sufficient to activate signaling. Therefore, TrkA oligomers are fully competent to respond to NGF, and initiate signaling after NGF binding.

The experimental evidence underlying the current model of ligand-induced TrkA receptor dimerization is derived primarily from chemical cross-linking studies (Jing et al., 1992; Meakin and Shooter, 1991; Schlessinger and Ullrich, 1992). Similar cross-linking studies of the platelet derived growth factor receptor β (PDGF- β) and the epidermal growth factor receptor (EGFR) also suggested that these receptors exist in the plasma membrane as monomers that dimerize upon addition of ligand (Cochet et al., 1988; Heldin et al., 1989; Inui et al., 1993). However, the use of new biophysical methods such as fluorescence resonance energy transfer (FRET) and image correlation spectroscopy (ICS) have challenged this model. In contrast to the chemical cross-linking data, FRET analysis demonstrated that EGF receptors exist in a pre-oligomerized state in the plasma membrane of A431 cells (Gadella and Jovin, 1995), and ICS analysis of non-transformed human fibroblasts demonstrates that PDGF- β receptors are pre-aggregated as tetramers in the absence of ligand (Wiseman and Petersen., 1999). Our finding that TrkA receptors are pre-oligomerized is consistent with these observations. In the future, it will be important to apply these other biophysical techniques to study TrkA aggregation in mammalian cells.

The hypothesis that TrkA is a constitutive oligomer in the oocyte can explain several observations that are difficult to reconcile with the notion that TrkA receptor dimerization is sufficient for signaling. A dimer composed of NGF conjugated to NT-4 activates transphosphorylation of TrkA receptors and initiates biological responses, even though NT-4 (at physiological concentrations) does not bind to TrkA receptors (Treanor et al., 1995). This strongly suggests the NGF/NT-4 heterodimer activates pre-formed TrkA

receptor dimers. Similarly, small peptide monovalent ligands, can activate TrkA receptors and promote neurite outgrowth in PC12 cells (Maliartchouk et al., 2000a) and primary embryonic dorsal root ganglion (DRG) neurons (Maliartchouk et al., 2000a), prompting the hypothesis that small molecule peptidomimetics stabilize TrkA receptor conformations that promote receptor activation.

Recent studies suggest that TrkA receptors must be in close proximity to each other in the plasma membrane before ligand binding. TrkA receptors bearing deletions and point mutations within the first or second immunoglobulin-like binding domains are constitutively phosphorylated and biologically active in the absence of NGF (Arevalo et al., 2000). The first immunoglobulin-like domain of TrkA appears to play a key role in regulating this ligand-independent activation (Zaccaro et al., 2001). To explain these findings, Arevalo et al. have suggested that there is a balance of attractive and repulsive forces between TrkA receptor monomers in the membrane, which is altered by NGF binding (or mutations within the ligand binding domain of TrkA) (Arevalo et al., 2000). Our findings are also not at odds with the observation of Woo and colleagues, who demonstrated that NGF promotes dimerization of the soluble extracellular domain of TrkA (Woo et al., 1998). Rather, they argue for the importance of the transmembrane domain in promoting ligand-independent TrkA oligomerization. Consistent with this, most growth factor receptor tyrosine kinases, including TrkA receptors, contain a conserved transmembrane dimerization motif (Sternberg and Gullick, 1990) that may enable them to form inactive dimers in the absence of ligand (Jiang and Hunter, 1999). Our study suggests that TrkA receptors may be activated in a similar fashion to other tyrosine kinases receptors, such as the ErbB2/neu and EPO receptor, for which dimerization is necessary, but not sufficient, to activate signaling (Burke et al., 1997, 1998; Jiang and Hunter, 1999; Livnah et al., 1996, 1998, 1999; Remy et al., 1999). Precise rotational coupling of receptor monomers induced by ligand-binding may be required to initiate transphosphorylation of critical tyrosine residues that are required for signal transduction (Jiang and Hunter, 1999).

In summary, the unique characteristics of the *Xenopus* oocyte expression system allowed us to use combined functional, biochemical, and structural methods to study the role of NGF binding in TrkA receptor dimerization. We conclude that, in the absence of NGF, TrkA receptors form oligomers in the plasma membrane of oocytes. Therefore, activation of signal transduction via the TrkA receptors likely involves conformational changes that are induced by NGF binding to pre-formed oligomeric receptor complexes. In the future, it will be important to resolve the nature of the conformational changes that accompany NGF binding, and determine whether constitutive TrkA receptor complexes are present in the plasma membrane of mammalian cells as well.

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