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IDENTIFICATION OF NGF RECEPTOR IN CHROMATIN OF MELANOMA CELLS USING MONOCLONAL ANTIBODY TO CELL SURFACE NGF RECEPTOR[1]

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SUMMARY. A 230 KDa species of Nerve Growth Factor (NGF) receptor was immunoprecipitated from EcoRI-digested chromatin of melanoma cells using a monoclonal antibody to the 75 KDa cell surface NGF receptor. The chromatin NGF receptor was shown to exist tightly bound to DNase II-sensitive sequences which, upon growth factor binding, became resistant to DNase II digestion.

Human melanoma cells express surface NGF receptor at much higher levels than do nevus cells or melanocytes (1). The cell surface NGF receptor of melanoma cells has been immunoprecipitated with anti-NGF receptor monoclonal antibodies (MAb) and characterized as a 75 KDa protein (1), which may also exist as an oligomer of 180-200 KDa (2). Recently we have shown that NGF binds to those melanoma cells that have NGF surface receptor, is taken up into the nucleus, and binds in non-degraded form to the chromatin (3). In this report, we demonstrate the presence of the chromatin NGF receptor, which is immunoprecipitated by the same MAb (ME 20.4) as the cell surface receptor but is of different molecular weight.

MATERIALS AND METHODS

Cell lines. Human melanoma cell line HS 294 (4, 5) and colorectal carcinoma SW 1116 (6, 7) were grown in minimal Eagle's medium supplemented with 10% bovine serum.

Monoclonal antibodies. Anti-NGF receptor MAb ME 20.4 was generated by the fusion of mouse myeloma cells with splenocytes of Balb/c mice immunized with WM 245 melanoma cells which express NGF receptor (1).

Incubation of cells with [^{125}I]NGF. Cells grown as monolayers were incubated as described previously (3) for 1, 24, or 48 hr in medium containing [^{125}I]NGF (10 ng/ml, spec. act., 22.5 cpm/pg) labeled by the lac-

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toperoxidase method (8). After incubation, cells were washed 3 times with PBS and chromatin isolated.

Labeling of chromatin protein. Chromatin proteins were incubated with [^{35}S]methionine (30 µCi/ml, spec. act. 1000 mCi/mmol) for 18 hr as described (9).

Chromatin isolation. Chromatin was isolated as described previously (3, 10).

Digestion of chromatin with DNase II. Chromatin was digested for various times at 37°C with DNase II [1 enzyme unit/500 µg of chromatin/ml in 25 mM sodium acetate (pH 6.6)] according to Gottesfeld (11) with modification (3).

Digestion of chromatin with the restriction endonuclease. Chromatin was resuspended to 500 µg/ml in buffer (50 mM NaCl, 100 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.01% BSA) optimal for EcoRI activity and digested with EcoRI (1-2 units/µg DNA) overnight at 37°C. EcoRI was used since it is active at the low salt concentrations optimal for immunoprecipitation and has frequent cut sites in human DNA. Insoluble chromatin was pelleted in a Microfuge at 15,000 x g for 20 min and discarded. The supernatant was designated spn 1.

Immunoprecipitation of chromatin NGF receptor. The immunoprecipitation procedure of Dorbic and Witting (12) was used with some modifications. Spn 1 at O.D. = 0.2 (A_{260}) was adjusted to 10 mM EDTA, 0.25% BSA and incubated with 25 µl of MAb ME 20.4 for 3 hr at room temperature. P3x63Ag8 supernatant was used as control. After the incubation, the mixture was incubated with formalin-fixed Staphylococcus aureus for 1 hr at 4°C and pelleted in a Microfuge at 15,000 x g for 1 min. The supernatant was designated spn 2. The pellet was washed twice with 50 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl (pH 7.4), 0.25% BSA, suspended in 50 µl of 1% SDS, 2 mM EDTA, 1% 2-mercaptoethanol, 30% glycerol, 0.05% bromophenol blue, 50 mM Tris-HCl (pH 8.0) and incubated for 3 min at 95°C. After centrifugation, the supernatant contained the dissociated proteins.

Electrophoresis. Electrophoretic analysis of proteins was performed in 7.5 or 5% SDS-polyacrylamide gels as described by Laemmli (13). Gels were stained with Coomassie blue, incubated with autoradiography enhancer, dried and autoradiographed for various times (up to 48 hr).

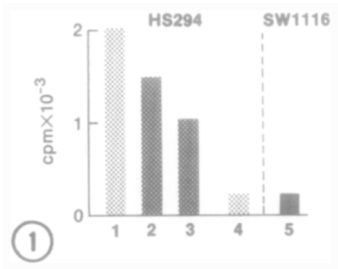
Solid phase radioimmunoassay. Chromatin adsorbed to the wells of polyvinyl chloride microtiter plates was incubated with MAb ME 20.4 and subsequently with ^{125}I-labeled rabbit anti-mouse IgG as described previously (3).

In competitive binding assay, chromatin adsorbed to the wells (1 µg/well) was preincubated with NGF (70 ng/well).

Binding of [^{125}I]NGF to isolated chromatin. Chromatin was adsorbed to the wells of microtiter plates (1 µg/well) as described (3). [^{125}I]NGF at concentrations from 0.1 to 40 ng/ml was added to each well and incubated for 2 hr at room temperature. After washing with PBS, bound radioactivity was measured. In competitive binding assays, chromatin adsorbed to the wells was preincubated with unlabeled NGF (700 ng/ml) or with ME 20.4, washed with PBS and then incubated with [^{125}I]NGF (3).

RESULTS

Binding of ME 20.4 to chromatin. MAb ME 20.4 bound specifically to chromatin of HS 294 melanoma cells, which express the surface NGF receptor, but



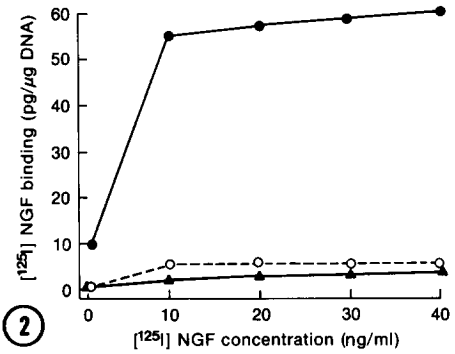


Figure 1. Chromatin binding of anti-NGF receptor MAb ME 20.4. Bars 1-4 represent binding to melanoma cell line HS 294 total chromatin, 0.35 M NaCl-extracted chromatin, 2 M NaCl-extracted chromatin, and chromatin preincubated with unlabeled NGF (70 ng/μg chromatin), respectively. Bar 5 shows binding to chromatin of colorectal carcinoma cell line SW 1116.

Figure 2. Binding of [^{125}I]NGF to HS 294 melanoma cell chromatin (•—•) and to chromatin preincubated with ME 20.4 (o---o) or with unlabeled NGF (▲—▲).

not to chromatin of colorectal carcinoma SW 1116 cells, which do not express this receptor (Fig. 1). Even after extraction of chromatin with 0.35 M and 2 M NaCl, binding of ME 20.4 was still detected, although at levels decreased by 50% (Fig. 1). Thus, the chromatin binding site for NGF seems to be tightly bound to the DNA.

In competition assay, binding of ME 20.4 to the chromatin was completely inhibited by NGF (Fig. 1). Like unlabeled NGF, ME 20.4 inhibited binding of [^{125}I]NGF to isolated chromatin by 90% (Fig. 2).

Immunoprecipitation of NGF receptor from chromatin. Fig. 3A shows the electrophoretic profile of [^{35}S]methionine-labeled chromatin proteins of intact chromatin and of EcoRI-digested chromatin. Several [^{35}S]methionine-labeled chromatin proteins ranging from greater than 200 to less than 30 KDa were resolved from intact chromatin (lane 1). In the EcoRI-digested chromatin (spn 1, lane 2), a prominant band at approximately 230 KDa and several proteins of less than 70 KDa were observed. Longer exposure revealed most chromatin proteins (not shown). After immunoprecipitation of spn 1 with MAb ME 20.4, only the 230 KDa protein was specifically detected (lane 3). This protein was not revealed after immunoprecipitation with control supernatant P3X63Ag8, even in overexposed autoradiograms (lane 4), but it was observed in the fraction remaining after precipitation of spn 1 with P3X63Ag8 supernatant (lane 5). After immunoprecipitation with MAb ME 20.4, the 230 KDa band was visible in spn 2 only after prolonged exposure (not shown).

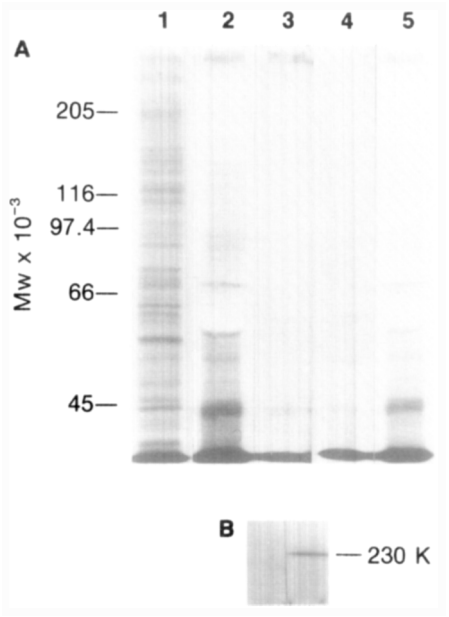


Figure 3. (A) SDS-PAGE (7.5% polyacrylamide) of [^{35}S]methionine-labeled proteins of intact (lane 1) and EcoRI digested chromatin (lanes 2-5) of HS 294 melanoma cells; lane 2: total proteins of digested chromatin (spn 1); lane 3: fraction immunoprecipitated with ME 20.4; lane 4: fraction immunoprecipitated with control supernatant P3X63Ag8; lane 5: fraction remaining after precipitation of spn 1 with P3X63Ag8 (spn 2).

(B) Coomassie blue staining after SDS-PAGE (5% polyacrylamide) of the fraction immunoprecipitated with ME 20.4 (spn 1) (lane 2) or with P3X63Ag8 (lane 1).

Coomassie blue staining after immunoprecipitation of spn 1 with ME20.4 (Fig. 3B, lane 2) or with P3X63Ag8 (Fig. 3B, lane 1) revealed the 230 KDa protein only in the ME20.4 immunoprecipitate.

Chromatin digestion with DNase II. To determine whether the chromatin NGF receptor is localized in DNase II-sensitive or -insensitive chromatin regions, chromatin isolated from HS 294 cells incubated for 1, 24 or 48 hr with [^{125}I]NGF was treated with DNase II until a limit digest of 12-20% DNase II-sensitive sequences was obtained. After 1 hr of incubation, approximately 65% of chromatin-bound [^{125}I]NGF (nonspecific release 15%) was found in the DNase II-sensitive, Mg^{2+}-soluble fraction, containing actively transcribed, preferentially digested sequences (Fig. 4). Thus, the chromatin NGF receptor appears to be localized in DNase II-sensitive chromatin. After 24 and 48 hr of incubation, DNase II digestion did not release [^{125}I]NGF (Fig. 4), indicating that the NGF binding regions became DNase II-resistant.

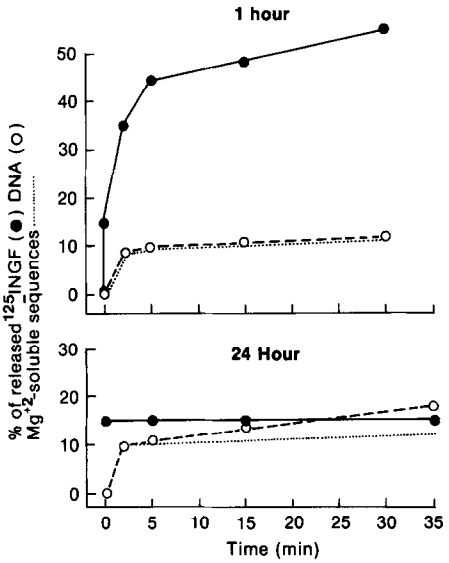


Figure 4. Kinetics of [^{125}I]NGF release during DNase II digestion.

DISCUSSION

The data above indicate that HS 294 melanoma cells, which express elevated levels of the 75 KDa cell surface NGF receptor, also express a 230 KDa NGF receptor species in the chromatin. ME 20.4, derived in mice immunized with intact melanoma cells (1), detects both of these NGF receptor species. It is conceivable that the 230 KDa chromatin receptor represents an oligomeric form of the 75 KDa species present also as a disulfide-stabilized oligomer on the surface of A875 but not HS 294 melanoma cells (1, 2). However, the 230 KDa form was identified in the presence of 2-mercaptoethanol. The 230 KDa form is efficiently labeled with [^{35}S]methionine, whereas the 75 KDa receptor is not (1, 2). Finally, unlike the surface receptor, which shows molecular weight heterogeneity (1, 2), the 230 KDa chromatin receptor appears to be homogeneous. Thus, we conclude that the 230 KDa chromatin receptor and the 75 KDa surface receptor represent distinct proteins.

Binding of ME 20.4 to the chromatin receptor was competitively inhibited by NGF, whereas according to Ross et al. (1) and Grob et al. (2), NGF did not inhibit ME 20.4 binding to the surface receptor. Therefore ME 20.4 and NGF seem to recognize the same binding site of the chromatin receptor but different sites in the case of surface receptors.

The chromatin NGF receptor is tightly bound to DNA, resisting extraction with 0.35 M and 2 M NaCl. To eliminate the need for high salt concentration (2 M NaCl-5 M urea) in dissociating chromatin proteins from DNA, chromatin was partially digested with EcoRI and the soluble restriction

fragment-protein complexes were used for immunoprecipitation. An analogous approach was used successfully by Dorbic and Whitting (12), who immunoprecipitated HMG-17 containing nucleosomes from chromatin digested with the micrococcal nuclease.

The intracellular action of peptide growth factors such as NGF, EGF, and PDGF (3) to some extent resembles that of steroid hormones (14-16). Steroid hormones penetrate different cells and are bound to specific cytoplasmic receptors of target tissues. The steroid hormone-receptor complexes penetrate the nucleus and are bound to specific chromatin acceptor sites generated by tissue-specific nonhistone proteins and specific sequences of homologous DNA (14, 15). Growth factors are recognized by specific surface receptors and are internalized (20-22). It is not yet known whether growth factors dissociate from the surface receptor before entering the nucleus or whether they remain bound to the surface receptor. Binding of glucocorticoid receptor complexes protects specific DNA domains from nuclease action (15). In the present study, we observe that NGF receptor is localized in DNase II-sensitive chromatin regions, which, after NGF binding, become DNase II-resistant. The same effect has been observed in the case of PDGF and EGF (3).

The chromatin NGF receptor is present in elevated amounts in HS 294 melanoma cell chromatin and is also detected in lower amounts by MAb ME 20.4 in A875 melanoma cells (unpublished data) and in SW 707 colorectal carcinoma cells (3), which expresses surface NGF receptor. However, the chromatin NGF receptor is not detected in SW 1116 colorectal carcinoma (Fig. 1) or cervical carcinoma SW 756 cells (unpublished data), which do not express surface NGF receptor (3). Thus, the chromatin NGF receptor may be involved in the intracellular action of this growth factor and may play a role in tumor transformation.

The two functional domains of the steroid receptor (DNA binding and steroid binding) exhibit homology with the oncogene v-erb A (20, 21). We do not exclude the possibility that 230 KDa chromatin NGF receptor may also be an oncogene product.

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