Molecular Cloning of Nonspecific Cross-reacting Antigens in Human Granulocytes*

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To clarify the molecular structures of nonspecific cross-reacting antigens (NCAs), a family of glycoproteins antigenically related to carcinoembryonic antigen (CEA), in human granulocytes, we have screened a cDNA library of human leukocytes using a cDNA probe for the N-terminal domain (domain-N) of NCA-50, an NCA species in tumor cells. In 95 positive clones randomly selected, we identified six NCA or NCA-related cDNA clones including NCA-50, biliary glycoprotein a, and W272 (CGMG) which have previously been reported, and three new clones, W236, W264, and W282, encoding three novel NCA species. W236 and W264 consist of a domain-N, a putative transmembrane domain, and a possible cytoplasmic domain. The domain-N of W264 is 89% similar to that of NCA-50 at amino acid level, whereas the domain-N of W236 is only 49 and 43% similar to those of NCA-50 and pregnancy-specific β,-glycoprotein-11 (PSG11), respectively, indicating that W236 belongs to a new subfamily within the CEA family. The third clone W282 encodes a protein consisting of a domain-N virtually identical to that of W264 and a short hydrophilic C-terminal domain. W264 and W282 seem to be derived from a single gene by alternative splicing of RNA. These three new species are particularly unique in respect that they lack the repetitive immunoglobulin-related domains that have been universally found in the human CEA gene family members. The biochemical and immunochemical properties of the recombinant proteins of these cDNA clones, however, did not coincide with those of six NCA species previously identified in granulocytes at protein level, suggesting that, in granulocytes, there exist at least 12 NCA or NCA-related species whose expression is under complex control.

Nonspecific cross-reacting antigen (NCA)* was initially identified in normal lung, spleen, and colon tumors (1, 2) as a cross-reacting antigen of CEA, which is one of the most useful tumor markers. A number of CEA-related antigens have since been detected in various human tissues as reviewed by Thompson and Zimmermann (3). NCA is currently used as a common name for a group of heterogeneous glycoproteins with diverse molecular sizes from 50 to 160 kDa, which have been found in normal or malignant colon, lung, spleen (4–7), granulocytes (8–11), and monocytes (8, 10). The molecular identity of these NCA molecules has been rather controversial.

The structure of NCA has been analyzed in comparison with that of CEA, which is composed of seven immunoglobulin-related domains (an N-terminal domain (domain-N) and six repetitive domains (A1–3 and B1–3)) and a domain-M that is post-translationally replaced with a glycosyl-phosphatidylinositol anchor (12–18). The primary structures of two NCA species, NCA-50 and W272 (CGMG), have so far been determined by screening of cDNA libraries of tumor cell lines (19, 20) and of normal (21) or leukemic leukocytes (22), respectively. The peptide of NCA-50 consists of four domains: a domain-N, two repetitive domains (A1 and B1), and a domain-M which is also replaced with a glycosyl-phosphatidylinositol anchor (23). The peptide of W272 has the same domain structure as that of NCA-50 but has a different sequence with about 70% similarity (21).

We have previously identified six NCA species at protein level in granulocytes, which were indicated to be distinct species from NCA-50 on the basis of their peptide size and immunoreactivity (11, 24). In the present study to elucidate the molecular structures of these NCA species in granulocytes, we screened a cDNA library of peripheral leukocytes, and identified six NCA or NCA-related cDNA clones, including NCA-50, W272 which has already been analyzed (21), BGP a, another CEA-cross-reacting antigen (25, 26), and three new clones encoding three novel NCA species. We report here the full sequences of the three new cDNA clones. In addition, the biochemical and immunochemical properties of the recombinant proteins of these cDNA clones expressed on mammalian cells were examined for comparison with the six NCA species identified at protein level in granulocytes.

EXPERIMENTAL PROCEDURES*

RESULTS

Nucleotide and Deduced Amino Acid Sequences of the Three Newly Identified NCA Clones—Ninety-five cDNA clones were

* Portions of this paper (including "Experimental Procedures," and Figs. 1, 2, 4–8, and 10–12) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

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‡ The abbreviations used are: NCA, nonspecific cross-reacting antigen; CEA, carcinoembryonic antigen; PSSG, pregnancy-specific β,-glycoprotein; BGP, biliary glycoprotein; CHO cells, Chinese hamster ovary cells; mAb, monoclonal antibody; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; UTR, untranslated region; aa, amino acid.
randomly selected from about 200 clones that hybridized with the cDNA probe corresponding to the domain-N of NCA-50. Portions of them were mapped with restriction endonucleases.

On the basis of the similarity of the restriction enzyme sites for Ncol, NsiI, PstI, and BglII to those of NCA-50 (Fig. 1), two of the positive clones, designated W264 and W282, were first chosen for further analyses. The sequence analysis of the clone W264 revealed that the 1,259-base pair insert contained an open reading frame of 252 amino acid residues (Fig. 2). By comparison with the primary structures of NCA-50 and other antigens in the CEA gene family, the sequence stretches from -34 to -1 and 1 to 108 were found to correspond to those of the signal peptide and domain-N, respectively, of the CEA family members. Fig. 3 shows the alignments of the domain-N sequences of the W264 and other members of the CEA family. The domain-N of the W264 are about 90% similar in amino acid sequence to those of NCA-50 (19, 20), BGPa (25, 26), and CEA (12-14), and P5G11 (44, 45). Identical residues are indicated by dashes. Potential N-glycosylation sites are underlined. Percent at the end of each sequence indicates the sequence similarity with W264 and that in parentheses with W236.

The nucleotide and deduced amino acid sequences of W264, W282, W236, and the CEA gene family members. The deduced amino acid sequence of the entire domain-N of W264 is aligned with those of W282, W236, and the antigens of the CEA gene family: NCA-50 (19, 20), W272 (21), CGM2, and the antigens of the CEA subfamily CGM (44, 45). It shows about 70% similarity to the domain-N of the CEA family members (Fig. 3). The hydropathy profiles of the W264 and W236 proteins are strikingly similar to each other (Fig. 4) despite the substantial difference in the sequence. Interestingly, the sequence of the putative transmembrane region is almost identical to that of the domain-N of W264, which is about 56% similar to that of BGPa (Fig. 8). Some identities are observed among the sequences of the CEA and B family members (46).

Other NCA-related Clones Detected in the 95 cDNA Clones—In addition to these three novel cDNA clones and W272 previously reported (21), we identified, in the 95 cDNA clones positive with the NCA probe, several clones that encode NCA-50 and BGPa on the basis of the restriction enzyme maps and sequencing data (not shown). Frequency of each NCA-related clone was examined by dot hybridization using the oligonucleotide probes specific for W264/W282, W236, W272, NCA-50, and BGPa, and found to be about 50, 5, 3, 3, and 25%, respectively. The remaining clones were reactive with none of the probes. Although these results may not directly reflect their frequency at mRNA level due to the possible biases in cDNA synthesis, proliferation, and detection efficiency, W264 or W282, or both, are possibly the major
transcripts in these NCA-related species in granulocytes. It should be noted that because all these clones were isolated using the probe corresponding to the domain-N of NCA-50 and analyzed with oligonucleotide probes as described under “Experimental Procedures,” mRNA encoding those putative CEA family members which lack domain-N, if any, might not be detected. The domain structures of the proteins encoded by the six clones identified in the library were schematically shown in Fig. 9.

Biochemical and Immunochemical Properties of the Recombinant Proteins of the NCA-related cDNA Clones—We have previously identified six NCA glycoproteins in granulocytes, which were different from NCA-50 and from one another in molecular size, membrane binding mode, or immunoreactivity (24). In the present study, we prepared the recombinant proteins of W264, W282, W236, W272, and BGPa, and compared their properties with those of the NCAs identified at protein level. For W264, W282, and W236, COS-1 cells were transfected with the expression vectors pBSG-W264, pBSG-W282, and pBSG-W236, respectively. For W272 and BGPa, CHO cells were transfected with pdKCR-dhfr-W272 and pdKCR-dhfr-BGPa, respectively.

The transfectants of W264, W236, and BGPa were surface-labeled with 35[35S]methionine, and the transfectants of W282 were metabolically labeled with [35S]methionine because the W282 protein was expected to be secreted. Immunoprecipitates from cell lysates or spent culture medium were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The results for W272 were reported in a previous paper (21).

The transfectants of W264 expressed a component of about 35 kDa reactive with polyclonal anti-NCA (Fig. 1OA, lane 2). By treatment with N-glycanase, the molecular mass of the W264 protein was reduced to about 27 kDa (Fig. 1OA, lane 3). As predicted from its sequence, the transfectants of W282 released an about 28-kDa component reactive with the anti-NCA into the medium (Fig. 1OB, lane 2). By incubation of the transfected cells with tunicamycin, the unglycosylated W282 peptide of about 20 kDa was detected in the cells (Fig. 1OB, lane 6), and in less amounts, in the medium (Fig. 1OB, lane 3). In spite of its low sequence similarity (about 50%) of the domain-N to other CEA family members, the recombinant protein of W236 was reactive with the anti-NCA, as identified as a diffuse band of about 40 kDa (Fig. 11A, lane 2). When this product was treated with N-glycanase, its apparent mass was reduced to about 28 kDa (Fig. 11A, lane 3). These W264, W282, and W236 proteins were also reactive with a polyclonal anti-CEA antibody (not shown). The transfectants of BGPa produced a component of about 140 kDa reactive with the polyclonal anti-CEA (Fig. 11B, lane 2), which gave a mass of about 70 kDa after deglycosylation with N-glycanase (Fig. 11b, lane 3). As can be seen in Table I, some of the unglycosylated peptides of the recombinant proteins were very similar in molecular size to those of the granulocyte NCAs previously reported; for example, NCA-90 and W264 or W236. However, they were different in membrane binding mode or immunoreactivity (see below).

Antigenic reactivities of these recombinant proteins were further analyzed with three mAbs, F34–187, F36–54, and F106–88, which have different specificities against the peptide moieties of CEA and NCA-50 (41). Upon enzyme immunooassays, both the transfected cells expressing the W264 and W272 proteins revealed a reactivity with F34–187 but not with the other two mAbs, F36–54, and F106–88 (Fig. 12, A and B). The cells expressing BGPa were also reactive with F34–187, and they showed a weak but significant reactivity with F36–54 (Fig. 12C). However, the cells expressing the W236 protein were reactive with none of the three mAbs (Fig. 12D) under the conditions that the polyclonal anti-CEA showed a positive reaction (not shown).

These results are summarized in Table I with the data obtained in a previous study (11, 21, 24, 41). Surprisingly, none of the recombinant proteins revealed a complete agreement in their properties with those of the NCAs detected at protein level in granulocytes.

**DISCUSSION**

In a cDNA library of human leukocytes pooled from about 100 healthy volunteers, we newly identified three clones, W264, W282, and W236, which encode three novel glycoproteins belonging to the CEA gene family. The domain organization of the three proteins was found to comprise an N-terminal domain and either transmembrane and cytoplasmic domains or a C-terminal domain. All the proteins in the human CEA family thus far reported consist of a domain-N and two to six repetitive domains which are related to the domains of immunoglobulin molecules (15, 16). The proteins encoded by the three newly identified cDNA clones are the first examples of the human CEA-related proteins that lack the repetitive domains.

The domain-N sequence of the W264 protein is about 90% similar to those of the CEA subfamily members, NCA-50, CEA, and BGPa, but less similar (about 45%) to those of the members of the other subfamily PSEG, indicating that W264 and W282 may be categorized into the CEA subfamily.

The overlapping sequences of W264 and W282 are strikingly similar suggesting that they are most likely derived from the same gene, the differences being polymorphism (see Fig. 6). Apparently, the extra sequence in W282 is derived from another exon that is incorporated into W282 mRNA by alternative splicing. Actually, we have cloned a genomic DNA which carries sequence identical to this region except for two nucleotide substitutions. Interestingly, nucleotides 765–992 which follow the “domain-A1” coding region, and are regarded as a 5’-terminal part of introns in general for CEA family members (47, 48), are in the mRNA in this case.

It should be noted here that some of the cell-surface proteins and their soluble counterparts are generated by alternative splicing of RNA, e.g. the major histocompatibility complex antigens (49) and N-CAM (50), the members of an immunoglobulin supergene family. Apparently W264 and W282 are, respectively, membrane bound and soluble forms of the gene product generated by alternative splicing, which seems one of the major mechanisms generating the multiple forms of CEA family members (26, 47, 48).

The domain-N of W236 exhibits only 45–50% similarity to those of the other members of the CEA gene family. This is low compared with the similarities among the other CEA gene...
family members (60-90%). W236 may be classified into a subfamily other than the CEA and PSG subfamilies. Interestingly, the sequence of the membrane-spanning region of the W236 protein is almost identical to that of the W264 and 56% similar to that of BGPa, which may suggest the recent divergence of this region in W236 and W264. The cytoplasmic regions of these three proteins are also similar in the 5' half, but not in the 3' half, which may reflect their diverse biological functions.

Unexpectedly, none of the corresponding proteins of the cDNA clones W264, W282, W236, W272, BGPa, and NCA-50 coincides with any of the six NCA glycoproteins identified in granulocytes in biochemical and immunochemical properties. This suggests that, despite their existence at mRNA level, the corresponding proteins of these cDNA clones are poorly expressed in granulocytes, hence hardly detectable by the methods we used. The lack of a correlation between the levels of mRNA and the corresponding protein has been reported for some proteins, e.g. myeloperoxidase (51) and growth-related proteins (52); in these cases, inefficient translation or extremely rapid turnover of the proteins was suggested. The corresponding proteins of the cDNA clones identified in this study, including NCA-50, W272, and BGPa, may also be translated inefficiently. It is tempting to speculate that the translation of the mRNAs for the clones identified in this study would be transiently accelerated by some event in the life span of granulocytes. On the contrary, genes for the major species of NCA identified in granulocytes at protein level may be activated only at the early stage of cell differentiation, and the turnover of the proteins may be so slow as to remain in terminally differentiated granulocytes, as in the case of lactoferrin in granulocytes (53). This is supported by the fact that four NCA species identified on granulocytes by surface labeling with [*]I could not be identified by metabolic labeling (24). Although two of the four NCA species (NCA-95 and NCA-90) were indicated to be anchored to the cell membrane with a glycosyl-phosphatidylinositol moiety (24), the possibility should be considered for the other two species (NCA-160 and NCA-26) that they might be produced by some other tissues or cells and trapped by the cell-surface of granulocytes. When we classified the NCA-positive clones by dot hybridization using the oligonucleotide probes, we found a few clones with different lengths and restriction enzyme maps, which are yet unsequenced, in each group. Therefore, it is also possible that the NCA glycoproteins identified in granulocytes, particularly those detected by metabolic labeling, are coded by some of these unsequenced clones, whose sequencing are now underway. In any case, the results presented here may suggest the existence of at least 12 molecular species of NCA or NCA-like antigens whose expression seems to be under complex control in granulocytes.

The members of the CEA subfamily, CEA (39, 54), NCA-50 (39), BGPa (55), and W272 (40), were demonstrated to be able to mediate homophilic and/or heterophilic cell adhesion when expressed on hamster cells. It is therefore likely that the W264 or W236 protein exhibits some cell adhesion activity. If this is the case, the W282 protein, a possible soluble counterpart of the W264, might modulate the adhesion activity of W264. Thus, it is feasible that NCA species in granulocytes identified at gene level as well as at protein level play important roles in many biological activities of granulocytes through participating in cell-cell interactions.

Acknowledgments—We thank Miwa Sohda for technical assistance and Keiko Fukushima for secretarial assistance.

REFERENCES

cDNA Cloning for Granulocyte NCAs

EXPERIMENTAL PROCEDURES

cDNA Library Construction and Screening — Isolation of poly(A)-RNA from pooled human peripheral leukocytes was described in a previous paper (21). cDNA was prepared by using the cDNA synthesis kit (Amersham) and methylated with EcoRI methylase. 5' EcoRI-linearized cDNA was ligated to EcoRI-linearized pBR322 vector (Boehringer) and ligated to λ Zap II (Stratagene). The ligated cDNA was packaged into bacteriophages by using the GigaPack Gold kit (Stratagene). About 2 x 10^8 pfu were plated on E. coli HB101 and green colonies were picked. Isolated cDNA from plaques was inserted into Bluescript SK-(-) (Stratagene) as an SmaI-EcoRI fragment (22) for preservation. After the filters were transferred to a blocking solution containing 5% (w/v) nonfat dry milk, 1% N-ethyl-3-amino propyl ethylenimine, 0.1% SDS, and 0.1% SDS, they were hybridized over night at 65°C with a 32P-labeled NcOl-Ssil cDNA fragment (382 bp) corresponding to the domain-N of NCA-50 (19), which had been isolated by the random primer DNA labeling method (27) with the use of the DNA-labelling kit (Nichirei, Tokyo, Japan). After washing with 70% (v/v) in a solution of 2 x SSC and 0.1% SDS, the filters were exposed to X-ray film.

Molecular Cloning of Non-specific Cross-reacting Antigens (NCAs) in Human Granulocytes

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**Fig. 1** Restriction enzyme maps and strategies of sequencing for three cDNA clones, W264, W282, and NCA-50. (A) W264 (353 bp); (B) W282; (C) NCA-50; (D) NCA-50 for comparison with Ret (19). The arrows indicate the domain-N probe for NCA-50 tor comparison (from Ref. 19). The restriction enzyme sites for NCA-50 are indicated only for those shared by at least one of the three domains. The arrows indicate the domain-N probe for NCA-50 tor comparison (from Ref. 19). The restriction enzyme sites for NCA-50 are indicated only for those shared by at least one of the three domains.

cDNA Cloning for Granulocyte NCAs

by

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SUPPLEMENTARY MATERIALS TO:

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Experimental procedures

**Sodium Deoxyribonucleoside and DTT Hybridization** — According to the sequences determined by the dideoxy chain termination method (28) and the DNA sequencing kit (United States Biochemical). Enzymes used in these experiments were from Nichirei and Nippon Gene and Kakuno Shuzo.

Computer Analysis of cDNA and Protein — Nucleotide and protein sequences were analyzed by using the MacGene System (Becton) and the GENASYS System at Riken University Computer Center (31). Hydrophathy analysis was carried out in accordance with Kyte and Doolittle (32).

Synthesis of Oligonucleotides and DTT Hybridization — According to the sequences determined by the dideoxy chain termination method (28) and the DNA sequencing kit (United States Biochemical). Enzymes used in these experiments were from Nichirei and Nippon Gene and Kakuno Shuzo.

**Fig. 2** Nucleotide and deduced amino acid sequences of the cDNA clones W264. Nucleotides and amino acids are numbered as described above. Vertical dotted lines indicate the potential starting positions of the remaining domain (TM, transmembrane; CT, cytoplasmic tail). Bold characters indicate the boundaries of exons. Potential glycosylation sites are indicated by thick underlines. Amino acids indicate the stop codon. Polyclonal antibody signal is boxed.
Fig. 4 Hydrophathy profiles of the corresponding proteins of W264, W282, and W36. Hydrophathy values obtained according to Kyte and Doolittle (26) were plotted with respect to positions in the deduced amino acid sequences of W264, W282 (A), and W36 (B). The profile for the leader domain and domain-N of W36 was virtually identical to that of W264 and omitted in this figure. The profile for domain-C of W264 was depicted by a lighter line. The domain structures of related proteins were estimated according to the predicted structures of the gene family antigens are shown above the hydrophathy profiles. L, leader peptide; N, domain-N; TM, transmembrane domain; CYT, cytoplasmic domain.

Fig. 5 Nucleotide and deduced amino acid sequences of the cDNA clone W264. The sequences of the cDNA clone W264 are depicted as the legend for Fig. 2. A broken underline indicates the nucleotide sequence similar to that of the domain-A1 of NCA-50, and a thin underline indicates the sequence similar to that of the putative transmembrane domain. Asterisks indicate gaps for alignment. Identical residues are boxed. Numbers above and under the sequences indicate their positions in W264 and W282, and in BGPc, respectively. Boundaries of the domains are arbitrarily defined according to Barnett et al. (26).

Fig. 6 Schematic representation of sequence relatedness among W264, W282, and NCA-50. Wide and narrow columns show translated and untranslated regions, respectively. Domains are indicated with leader peptide, N (domain-N), A (domain-A1), B (domain-B), C (domain-C), TM (transmembrane domain), and CYT (cytoplasmic domain). Nucleotide positions are shown in parentheses. Sequence similarity (%) at nucleotide level is indicated between regions.

Fig. 7 Nucleotide and deduced amino acid sequences of the cDNA clone W282. The sequences are as described in the legend for Fig. 2.
cDNA Cloning for Granulocyte NCAs

Fig. 10 SDS-PAGE of the recombinant proteins of W264 and W272. (A) COS-1 cells transfected with the expression vector pSG5-W264 were surface-labeled with [35S]methionine and analyzed by SDS-PAGE as described under "Experimental Procedures." Lane 1 is for molecular weight standards. (B) COS-1 cells transfected with the expression vector pSG5-W272 were metabolically labeled with [35S]methionine in the presence (lanes 3, 6, and 7) or absence (lanes 2, 4, and 5) of tunicamycin. Immunoprecipitates from spent culture medium (lanes 2-4) and cell lysates (lanes 5-7) were analyzed by SDS-PAGE as described under "Experimental Procedures." Arrows indicate the unglycosylated form of the W272 protein. Lane 1 is for molecular weight standards.

Fig. 11 SDS-PAGE of the recombinant proteins of W236 and BGPa. (A) Parental COS-1 cells (lane 1) or COS-1 cells transfected with the expression vector pSG5-W236 (lanes 2 and 5) were surface-labeled with [35S]methionine. Immunoprecipitates from cell lysates of the transfectants with rabbit anti-NCA were treated (lane 3) or untreated (lane 2) with endoglycosidase (20 units/ml) and analyzed by SDS-PAGE as described under "Experimental Procedures." A specific band was observed with normal IgG (not shown). Lane 4 is for molecular weight standards. (B) CHO cells transfected with the expression vector pcRCH-dfr-BGPa were surface-labeled with [35S]methionine. Immunoprecipitates from cell lysates of the transfectants with rabbit anti-CEA were treated (lane 3) or untreated (lane 2) with endoglycosidase (10 units/ml) and analyzed by SDS-PAGE. Lane 1 is for molecular weight standards.

Fig. 12 Reactivities of the recombinant NCA-related proteins with MAbs against CEA and NCA. COS-1 cells transfected with pSG5-W264 or W272 and CHO cells transfected with pcRCH-dfr-BGPa or W272 were examined for the reactivity with three MAbs, F34-187, F36-54, and F106-98, which have different specificities for CEA and NCA-50, by enzyme immunoassay as described under "Experimental Procedures." Values obtained for parental COS-1 or CHO cells were subtracted from those for transfected cells. Results are representative of three different experiments. A. W264; B. W272; C. BGPa; D. W236.