Type II Human Complement C2 Deficiency

ALLELE-SPECIFIC AMINO ACID SUBSTITUTIONS (Ser189 → Phe; Gly444 → Arg) CAUSE IMPAIRED C2 SECRETION*

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Type II complement protein C2 deficiency is characterized by a selective block in C2 secretion. The Type II C2 null allele (C2Q0) is linked to two major histocompatibility haplotypes (MHC) that differ from the MHC of the more common Type I C2 deficiency. To determine the molecular basis of Type II deficiency the two Type II C2Q0 genes were isolated and transfected separately into L-cells. Subsequent molecular biology, biostatic, and immunofluorescence studies demonstrated that C2 secretion is impaired in Type II C2 deficiency because of different missense mutations at highly conserved residues in each of the C2Q0 alleles. One is in exon 5 (nucleotide C566 → T; Ser189 → Phe) of the C2Q0 gene linked to the MHC haplotype A11,B35,DRw1,BFS,C4A0B1. The other is in exon 11 (G1930 → A; Gly444 → Arg) of the C2Q0 gene linked to the MHC haplotype A2,B5,DRw4,BFS,C4A3B1. Each mutant C2 gene product is retained early in the secretory pathway. These mutants provide models for elucidating the C2 secretory pathway.

The complement system consists of about 30 soluble and membrane proteins that constitute one of several important mediators of host defenses against microbial infection. The complement protein C2 is a M, 100,000 serine protease that functions in the classical activation pathway of the complement system. It is encoded by a 20-kb gene of 18 exons that is tightly linked to the homologous 6-kb gene encoding the complement protein factor B (1–3). Both genes comprise part of the class III gene cluster (4) located on the short arm of chromosome 6 between the HLA-D and HLA-B loci of the major histocompatibility complex (MHC) (5, 6).

Deficiency of the second component (C2D) is the most common genetic deficiency of the complement system. In populations of western European origin, the C2 null gene (C2Q0) frequency is about 1% (7, 8). Molecular heterogeneity in C2 deficiency was recently recognized based on expression of the protein in cell culture of fibroblasts from affected individuals (9). In Type I C2D, there is no detectable translation of C2-specific mRNA. Multiple C2D families from different geographic regions have been examined, and to date the Type I phenotype in each case results from a 28-bp deletion in the C2Q0 gene that removes 9 bp of the 3′-end of exon 6 and 19 bp of the 5′-end of the adjoining intron (10, 11). This deletion generates a mature C2 transcript from which exon 6 is deleted, creating a downstream premature stop codon and a failure to synthesize detectable C2 protein (10). Additionally, all C2Q0 genes examined containing the 28-bp deletion are linked to at least part of the same MHC haplotype/complotype (extended haplotype) A25,B18,C2Q0,BFS,C4A4B2,DRw2 (12, 13).

In contrast, Type II C2D is characterized by a selective block in C2 secretion (9) and is found in the context of two different MHC extended haplotypes that differ from that associated with Type I C2D, suggesting the possibility of more than one molecular mechanism leading to the secretory block. Accordingly, to examine the molecular genetic basis of Type II C2D, the two C2Q0 genes associated with the Type II extended haplotypes were isolated, transfected separately into L-cells, and the corresponding C2 cDNA sequenced. The data reported here establish that Type II C2D within the HLA haplotype A2,B5,DRw4 complotype C2Q0,BFS,C4A3B1 is due to a single missense mutation (nucleotide G1930 → A) leading to a Gly to Arg change at amino acid residue 444. Type II C2D in the context of the HLA haplotype A11,B35,DRw1 complotype C2Q0,BFS,C4A0B1 is due to a different missense mutation (C566 → T) leading to an amino acid change from serine to phenylalanine at residue 189. These single amino acid substitutions result in a marked inhibition of secretion of the respective C2 proteins, although the secretory block is more profound for the Arg444 mutant.

EXPERIMENTAL PROCEDURES

Type II C2-deficient Family—This family has been described in detail in our earlier report (9). The nuclear family members who are pertinent to this current study are shown in Fig. 1. Primary Fibroblast Cultures—Skin fibroblast cell lines were established from the Type II C2-deficient family members and from normal individuals as described previously (9). Fibroblasts were maintained at 37 °C in 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mm glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Isolation and Characterization of C2 Genomic Cosmid Clones—High molecular weight DNA was prepared from peripheral blood leukocytes obtained from individual II.8 (Fig. 1) as described previously (10). This Type II C2D individual contains both C2 alleles associated with Type II C2 deficiency (Fig. 1). The high molecular weight DNA was partially...
digested with Sau3A1 and used to prepare a genomic cosmid library as described in detail previously (14). Approximately 1 million recombinants were plated and screened in duplicate for clones containing the C2 gene by using a nick-translated (15) C2 cDNA as a probe (16). Of 11 clones that hybridized with the C2 cDNA, five were determined to contain the entire C2 and factor B genes by Southern analysis (17) using a cosmid library with EcoRI-NcoI fragments that hybridized to the 5' and 3'-ends of exons 1 and 18 of the human C2 and factor B genes, respectively. The five clones containing the entire human C2 gene were then examined for RFLPs, so that the clones could be separated into two groups, with each group corresponding to one of the two different Type II alleles. An EcoRI restriction site in intron 1 was found in the cosmid clone that was absent in the other four clones. A digest of the two groups was then selected for transfection and biosynthetic studies. The two clones were designated B and C, with C containing the EcoRI restriction site in intron 1. The genomic cosmid containing the entire normal C2 gene used in these studies was obtained from a library prepared using DNA isolated from the C2-sufficient sister of the Type I C2-deficient previously reported (9).

Transfection, Biosynthetic Labeling, and Immunoprecipitations— Murine kidney fibroblast L-cells (American Type Culture Collection, Rockville, MD) were grown to 50% confluence (5 × 10^6 cells/100-mm^2 dish) and transfected with genomic cosmid clones using the CaPO4 synthesis method of Gubler and Hoffman (22) and the reagents supplied by Stratagene (La Jolla, CA) and (23) human C2 cDNA (16). Phagemids were prepared, plated, and screened using a random-primed labeled PCR fragment of genomic cosmid clones and untransfected cells were transferred onto glass coverslips. Cells were incubated at 37°C for 48 h, rinsed in cold PBS, and permeabilized with 0.2% Triton X-100 in PBS (5 min) followed by incubation with 1:300 dilution of rat anti-human C2 antibody (Pierce) (diluted 1:5000 in PBS containing 3% bovine serum albumin, Tween 20, and incubated with biotin-conjugated rabbit anti-goat IgG purified goat IgG as a negative control (Pierce) (0.8 mg/ml, each). Cells were then reacted with avidin-phycocyanin-conjugated goat anti-rat IgG (diluted 1:400 in PBS containing 3% bovine serum albumin). All samples were reacted with 0.5 mg/ml normal goat IgG as a negative control. The samples were then washed and analyzed on a Cytofluorograph (Jandel Scientific) with 488 nm excitation.
RESULTS

Pedigree of Type II C2D Family—A diagram of the nuclear family with Type II C2D deficiency is shown in Fig. 1. The solid symbol, representing the Type I C2Q0 gene, is maternally inherited by two offspring (III-8, III-9) who are compound Type I/Type II C2-deficient. The father (II-8) is homozygous Type II C2D and contains both Type IIC2Q0 genes in association with the MHC extended haplotypes A2,B5,DRw4,BFS,C2Q0,C4A3B1 and A11,B35,DRw1,BFS,C2Q0,C4A0B1. Serum C2 levels were 76 ng/ml (4% of normal) and 57 ng/ml (0.2% of normal) for the Type II father (II-8) and propositus (III-9), respectively.

Biosynthesis of C2 and Factor B—A representative study of C2 and factor B biosynthesis in primary culture of fibroblasts from a normal and the Type II C2-deficient father with both Type II C2Q0 genes is shown in Fig. 2. C2 polypeptides of Mr 84,000,79,000, and 70,000 were detected in the cell lysates and C2 protein of Mr 95,000–100,000 in culture medium of [35S]methionine-pulse-labeled normal fibroblasts (Fig. 2, top panel, left two lanes), confirming previous studies of C2 synthesis and secretion (29). The same three intracellular C2 polypeptides were detected in the fibroblasts of the Type II C2D deficient father, but in markedly increased amounts (especially the Mr 84,000 and 70,000 forms), and a small amount of mature C2 protein of approximately normal size was detected in the extracellular medium (Fig. 2, middle two lanes, top panel).

To examine C2 biosynthesis from each of the C2D Type II genes, murine L cells were separately transfected with cosmid clones bearing the entire factor B and C2 genes corresponding to normal and each of the Type II C2Q0 genes (described in detail under “Experimental Procedures”). The latter were separable by the presence of an EcoRI RFLP in intron 1 of the C2 gene. The Mr 84,000 intracellular C2 protein was abundant in lysates of L cells transfected with cosmid clones derived from each of the Type II C2Q0 genes, but as in the primary fibroblasts only a trace amount of C2 protein was present extracellularly (Fig. 2, right two lanes, top panel). The apparent defect in secretion of C2 in Type II C2D fibroblasts and the transfectants is selective because factor B is synthesized and secreted normally in the deficient fibroblasts and the transfectants (Fig. 2, bottom panel).

Kinetics of C2 Secretion—To ascertain whether the limited recovery of C2 protein in extracellular medium was due to a decreased capacity of Type II C2D fibroblasts and the transfectants to secrete C2, a pulse-chase experiment was performed. In agreement with earlier reports (29), the results shown in Fig. 3 indicate that the Mr 84,000 C2 protein in normal fibroblasts was secreted with a half-time of about 1 h and that the Mr 79,000 and 70,000 C2 proteins remain cell-associated (Fig. 3, top panel). In contrast, the half-time for disappearance of the Mr 84,000 C2 protein in the Type II C2D fibroblasts was approximately 2–4 h, and only a trace amount of C2 was detected in culture medium at 8 h (Fig. 3, bottom panel). Fig. 4 shows data from pulse-chase studies of L-cells transfected with cosmids containing the normal and each Type II C2Q0 gene.
The biosynthesis of C2 in the normal and Type II transfected cells was similar to that for the corresponding primary cells. For example, in L-cells transfected with the normal clone, the disappearance of the Mr 84,000 intracellular C2 protein and appearance of the extracellular protein displayed kinetics similar to the primary cells. Additionally, the C2D Type II transfecants showed the same prolonged half-time of disappearance of intracellular C2 protein (half-time 2–4 h) as did the primary cells. In these experiments, the C2 in the extracellular medium was occasionally cleaved to C2a and C2b, shown by the C2a fragment of Mr 74,000 in the Normal and Clone C panels in Fig. 4. Factor B secretion kinetics (half-time approximate 60 min) were identical in all cell types and similar to previous reports (data not shown). Collectively, the biosynthetic experiments indicate that Type II deficiency is caused by mutations within the Type II C2 genes, since transfected cells duplicate the C2D phenotype of primary Type II fibroblasts.

Immunofluorescence of L-cells Transfected with C2 Genomic Cosmid Clones—Immunofluorescence studies of the transfected L-cells were performed as outlined under “Experimental Procedures.” Cells transfected with the normal C2 genomic cosmid clone showed a diffuse pattern of fluorescence throughout the cytosol of the cell (Fig. 5, panel a). In contrast, only perinuclear immunofluorescence was observed in L-cells transfected with either of the C2D Type II genomic cosmid clones (Fig. 5, panels c and d), suggesting distribution of C2 protein in the Golgi and/or rough endoplasmic reticulum of the C2D Type II transfectants. In addition, the overall fluorescence signal was approximately 10 times more intense in the C2D Type II transfectants compared with the normal transfecants (note exposure times in Fig. 5). The increased fluorescence observed in the C2D Type II transfectants is in accord with the immunoprecipitation data, indicating intracellular accumulation of the C2D Type II protein. The negative controls of untransfected L-cells (Fig. 5, panel b) and L-cells mock-transfected with vector alone (data not shown) showed no background immunofluorescence.

Northern Blot Analysis of C2 mRNA in C2D Type II Primary Fibroblasts and Transfected L-cells—To compare the mRNA transcribed from the normal and C2D Type II genes, RNA was isolated from primary fibroblasts and from L-cells transfected with normal and C2D Type II cosmid clones and subjected to Northern blot analysis as described under “Experimental Procedures.” The C2-specific mRNA detected in the C2D Type II primary fibroblasts (Fig. 6, lane 1) was similar in size (2.7 kb) and quantity to the C2 mRNA detected in normal skin fibroblast cells (data not shown). In addition, the L-cells transfected with normal (Fig. 6, lane 2) and both C2D Type II genomic cosmid clones (Fig. 6, lanes 3 and 4) expressed a major C2...
transcript of identical size (2.7 kb) as that seen in the primary fibroblasts. The transfectants contained 20–100-fold more C2 mRNA compared to the primary fibroblast cells. This increased C2 expression was expected, since the transfected L-cells each contained approximately 50 copies of C2 genomic DNA as determined by Southern blot analysis (described under “Experimental Procedures”). L-cells transfected with normal C2 and C2D Type II clones also expressed a less abundant smaller C2 transcript of 2.2 kb. Since this 2.2-kb transcript was seen in both normal and C2D transfectants but not in non-transfected L-cells (Fig. 6, lane 5), it is probably derived from aberrant splicing of the human C2 primary transcript by the mouse L-cells.

Sequence Analysis of the C2D Type II cDNA—To examine the C2 primary amino acid structure for mutations that would account for the impaired C2-specific secretion in C2D Type II cells, C2 cDNA were generated and sequenced using RNA from the L-cell transfectants. A full-length C2 cDNA clone was isolated from a cDNA library constructed using poly(A) mRNA harvested from L-cells transfected with cosmid clone B as described under “Experimental Procedures.” The nucleotide sequence of this cDNA was identical to published human C2 sequences except for a single base change (G → A) at nucleotide 1330. This substitution predicts a change in the amino acid at residue 444 from glycine to arginine and generates a PstI restriction site in exon 11 of the mutant C2Q0 gene (Fig. 7). The presence of this nucleotide substitution was confirmed by sequence analysis of PCR fragments generated from genomic DNA isolated from primary fibroblast cultures and peripheral white blood cells of the C2D Type II propositus (Fig. 1, III.9) and his father (Fig. 1, II.8).

The full-length C2 cDNA sequence corresponding to the other C2D Type II allele was delineated from overlapping subcloned cDNA fragments generated by RT-PCR using RNA isolated from the L-cells transfected with cosmid clone C (see “Experimental Procedures”). The nucleotide sequence of this cDNA was also identical to published human C2 sequences except for a single base change. In this case, a C → T substitution occurs at nucleotide position 566, resulting in a predicted serine to phenylalanine amino acid change at residue position 189, which is located in exon 5 of the C2Q0 gene (Fig. 7). The presence of this nucleotide substitution was confirmed by sequence analysis of PCR fragments generated from genomic DNA isolated from the father of the propositus. These results together with the biosynthetic data demonstrate that the T566 and A1330 nucleotide substitutions in exons 5 and 11 of the Type II C2Q0 genes are missense mutations that ultimately result in the synthesis of mutant full-length C2 precursor proteins. Because of each amino acid substitution (either Phe189 or Gly444), the C2 mutant precursor is retarded in transit through the normal C2 secretory pathway.

Determination of Type II C2Q0/HLA Linkage by RFLP Analysis—To determine the HLA haplotype linkage for each of the Type II C2Q0 genes, RFLP analysis was performed by PstI digestion of PCR generated C2 genomic DNA fragments of 431 bp that included exon 11 as described in the Experimental Procedures. The 431-bp genomic fragments were amplified using DNA isolated from the C2D Type II cosmid clones B and
C and from genomic DNA isolated from the mother, father, and propositus of the C2D Type II family (Fig. 8). As described above, the exon 11 missense mutation A1330 found in bone B generates a PstI RFLP. Therefore, as expected, digestion of the 431-bp fragment from cosmids bone B yields three bands of predicted size, one of 37 bp generated by a normal PstI site contained in exon 10, and bands of 217 and 177 bp generated by the PstI site created by the A1330 missense mutation (Fig. 8). Since neither the normal C2 gene nor the Type I C2Q0 gene contain the exon 11 missense mutation, digests of the PCR fragments from bone C and the heterozygous C2D Type I mother yielded as expected only the 37- and 394-bp fragments generated by the exon 10 PstI site (Fig. 8). Additionally, DNA from the homozygous C2D father who contains both Type II C2Q0 genes yields all four bands (394, 217, 177, and 37 bp), as would be predicted for someone containing the exon 11 missense mutation in one C2 allele. PstI digestion of DNA from the homozygous C2D propositus who contains a Type I and a Type II C2Q0 gene also yielded these four bands, thereby demonstrating that his Type II C2Q0 gene contains the exon 11 missense mutation. Moreover, this finding, together with previous tissue typing data (9), indicates that the extended haplotype linked to the Type II C2Q0 gene containing the exon 11 missense mutation is A2,B5,DRw4,BFS,C4A3B1 and that linked to the Type II C2Q0 gene containing the exon 5 missense mutation is A11,B35,DRw1,BFS,C4A0B1.

**DISCUSSION**

Type II C2 deficiency is characterized by a selective block in C2 secretion and has been found in the context of two different MHC haplotypes (9). Using L-cells transfected with the two separate Type II-associated C2Q0 genes, it is demonstrated here that C2 secretion is impaired in Type II cells because of two distinct C2Q0 allele-specific missense mutations that result in critical amino acid substitutions in the C2 protein structure. One missense mutation is in exon 11 (G1930 → A) in the Type II C2Q0 gene linked to the HLA haplotype A2,B5,DRw4, compleotype BFS,C4A3B1. This mutation results in a Gly444 → Arg substitution. The other missense mutation is in exon 5 (C566 → T) in the Type II C2Q0 gene linked to the HLA haplotype A11,B35,DRw1, compleotype BFS,C4A0B1 and results in a Ser189 → Phe substitution.

During the past decade, the molecular genetic basis of numerous protein deficiencies has been determined. The mutations that cause these deficiencies are of several different types and include various nonsense mutations, splice site mutations, transcriptional promoter sequence mutations, and missense mutations. As in the case of Type II C2 deficiency, recent studies have demonstrated that several protein deficiencies result from missense mutations that cause critical amino acid changes, which directly impair secretion of the affected protein. For example, secretory defects due to single amino acid substitutions have been reported to cause protein deficiencies of Type IIA von Willebrand factor (31), high molecular weight kininogen (32), α1-antichymotrypsin (33), human hepatic lipase (34), protein C (35), murine γ1 light chain (36), α1-antitrypsin (37), lysosomal α-glucosidase (38), and complement component C3 (39). In some of these cases, the molecular/cellular basis of the secretory defect has been examined. Some missense mutations appear to impair secretion by disrupting critical structural
domains that cause misfolding of the protein. In other cases, missense mutations do not cause large structural changes but instead alter important recognition determinants in the protein required for efficient processing, transport, and secretion. An example of the former case occurs in PiZZ protein required for efficient processing, transport, and secretion. missense mutations do not cause large structural changes but domains that cause misfolding of the protein. In other cases, missense mutations cause impaired C2 secretion are currently not known. The three-dimensional structure of C2 has not been determined; it is therefore difficult to predict what these two mutations might do to the overall structure of the C2 molecule. However, comparison of the murine and human C2 sequences indicate that both mutations are located in highly conserved regions of the C2 molecule, suggesting the importance of these regions in the normal expression of a functional C2 protein. For example, there is 74% overall amino acid identity between murine and human C2. In contrast, the phylogenetic identity proximal to the exon 5 and 11 missense mutations is much greater, with 100% identity observed in the 19 and 16 amino acids immediately surrounding the Phe<sup>189</sup> and Arg<sup>444</sup> mutations in exon 5 and 11, respectively, (2, 3). Moreover, exon 11, that encodes part of the C2 serine protease domain, is one of the most conserved exons in the C2 gene, with 94% sequence identity shared between the human and murine amino acid sequences. In addition to its location in a highly conserved region, the exon 11 Arg<sup>444</sup> mutation is only three amino acids upstream of a possible N-linked glycosylation site at Asn<sup>447</sup>. The charge change resulting from the Arg<sup>444</sup> substitution could disrupt the overall structure of this conserved region or inhibit proper glycosylation of Asn<sup>447</sup>. Either of these possibilities could affect interactions of the mutant C2 protein with resident endoplasmic reticulum proteins and cause retention in this compartment. The substitution of the small polar Ser<sup>189</sup> with a large aromatic nonpolar Phe residue in exon 5 could also disrupt structural features important in the secretion of C2, especially since the substitution occurs between two aromatic Tyr residues (Fig. 7).

Hereditary C2 deficiency is the most common complement deficiency in individuals of western European descent, with approximately 1 person in 10,000 being homozygous C2-deficient. More than half of homozygous C2D individuals have rheumatological disorders, including systemic lupus erythematosus (7, 42). In addition, many are predisposed to recurrent pyogenic bacterial infections (43). Current data indicate that the majority (＞93%) of C2 deficiency (C2Q0) genes contain the Type I mutation (28-bp partial gene deletion), and almost all Type I C2Q0 genes are linked to the extended haplotype HLA-A25,B18,BF5,C4A4B2,DRw2 (10, 11, 44). All remaining Type I C2Q0 genes are associated with parts of this haplotype, suggesting that the 28-bp deletion originated 600-1300 years ago with the complete haplotype (12). Recent reports have indicated that there is no apparent correlation with these different clinical manifestations and variations in the Type I C2Q0-associated HLA extended haplotypes (11, 45).

In contrast to Type I C2Q0 genes, it has been assumed that Type II C2Q0 genes are rare and comprise no more than the remaining 7% of C2D Caucasian individuals who do not contain the Type I mutation (10). However, the possibility that the abundance of Type II C2Q0 genes has been underestimated as the result of ascertainment bias should be considered. For example, the majority of C2D families have been discovered by the manifestation of one of the associated clinical problems in a homozygous Type I C2D family member. Since Type II C2D individuals contain some serum C2, it is possible that Type II homozygous C2D individuals do not develop clinical problems as readily as Type I homozygous individuals who lack detectable C2 in their serum. Now that the molecular genetic mutations causing Type II C2D have been delineated, it is possible to examine individuals who contain all or part of the two Type II C2D-associated MHC haplotypes for the Type II missense mutations. Such an investigation should yield a more definite
picture regarding the abundance of Type II C2Q0 genes and clinical manifestations associated with Type II C2D. Moreover, continued study of Type II C2D cells will reveal additional insights regarding folding, processing, and secretion of C2 as well as other secretory proteins in general.

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