Exon 6 Is Essential for Invariant Chain Trimerization and Induction of Large Endosomal Structures*

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Invariant chain (Ii) is a transmembrane type II protein that forms a complex with the major histocompatibility complex (MHC) class II molecules in the endoplasmic reticulum (ER). The membrane proximal luminal region of Ii is responsible for the non-covalent association with MHC class II molecules. Chemical cross-linking in COS cells was used to study the effect of luminal and cytoplasmic deletions on trimerization of Ii. We demonstrate that trimerization of Ii is independent of the cytosolic tail of Ii, whereas residues 162–191 (the sequence encoded by exon 6) in the luminal part of Ii are essential for trimer formation. Immunofluorescence studies of the transfected luminal deletion constructs show that the amino acids encoded by exon 6 of Ii are also essential for the induction of large endosomal vesicles. The data suggest that Ii must be in a trimeric form to modify the endosomal pathway.

The major histocompatibility complex (MHC)1 class II molecules are polymorphic heterodimers, consisting of an α- and a β-chain. Invariant chain (Ii) is a type II transmembrane protein that associates with the major histocompatibility complex (MHC) class II molecules in the endoplasmic reticulum (ER) (1, 2). The formation of the αβIi complex occurs by the sequential addition of one α- and one β-chain to a pre-existing core of trimeric Ii molecules (3, 4). This results in a stoichiometric 9-subunit complex (5). Following subunit assembly in the ER, the (αβIi)3 complexes traverse the Golgi complex. Approximately 1–2 h after biosynthesis, Ii molecules are sorted to and protein-tocytoplasmically processed in endosomal compartments (6–8), and MHC class II molecules with bound peptide are routed to the plasma membrane (9).

Invariant chain is composed of three domains: an N-terminal cytosolic tail of 30 amino acids, a membrane spanning region between residues 31 and 56, and a luminal C-terminal domain of 160 residues (159 in mouse) (10–14). An alternative form of the human Ii results from translation initiation at an upstream start codon, extending the cytosolic tail by 16 residues. Both human and mouse Ii contain an alternatively spliced exon in their luminal domains. These factors, together with various types of post-translational modifications, create numerous forms of the molecule (reviewed in Ref. 15).

Invariant chain contributes to the function of the MHC class II molecules in several ways. Invariant chain is responsible for efficient transport of MHC class II molecules out of ER into the endosomal pathway. This function is mediated by sorting signals in the cytosolic tail of Ii that direct the αβIi multimer to the endocytic pathway (16–19). The extra 16 N-terminal amino acid residues in the cytosolic tail of Ii include a strong ER retention signal (20), which also mediates retention of the associated MHC class II molecules. The biological significance of the retention of Ii in the ER remains to be elucidated. The cytosolic tail of wild-type Ii also contains information essential for the formation of large endosomal structures (7, 21). Induction of these large endosomal vesicles may be related to a delay in anterograde transport of fluid phase markers, as observed in the endosomal pathway of cells transfected with Ii (21, 22).

The region of Ii responsible for association with MHC class II molecules has been assigned to residues 81–109 in the Ii proximal membrane region (23). This association allows Ii to function as a chaperone for MHC class II molecules in the ER (24, 25) and to prevent premature peptide binding (26), thereby mediating transport to endosomal compartments and antigen presentation (18, 21, 27, 28).

In this study, we have investigated, by chemical cross-linking, how the multimerization of Ii depends upon the different parts of the molecule. By studying the intracellular distribution of various deletion mutants of Ii, we found that amino acids encoded by exon 6 of Ii were essential for the trimerization of Ii and the formation of large endosomal structures.

MATERIALS AND METHODS

Expression vectors and plasmid constructs—The pSV51L vector is a late replacement expression vector with an SV40 promoter, which gives high transient expression of several proteins in simian cells (16, 29). pSV51LI (p33 hIi) and p31 hIi have been described earlier (16). p35* hIi, in which the second methionine has been mutated, was a gift from E. Long, National Institutes of Health, Rockville, MD (30); 340 hIi (ΔN) and Δ26 hIi (ΔE), lacking amino acids 1–30 and 1–26, respectively, were a gift from J. Lipp and B. Dobberstein, Heidelberg (16). These three constructs were subcloned into the pSV51L vector.

The constructs encoding cDNA mIi (mcIi), genomic mIi, mIi Dα–81–127, mIi DΔ110–130, mIi DΔ110–161, mIi DΔ26–215, mIi DΔ153–208, and mIi DΔ192–212 have been described earlier (23). The plasmid constructs encoding mIi DΔ153–215 and mIi DΔ28–215 were made the same way, by first using a 5’ sequence derived from Ii cDNA fused via the SacI restriction endonuclease site within exon 2 to 3’ genomic sequences. Then, by deleting the sequence between AfII (exon 5) and DraIII (exon

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† The abbreviations used are: MHC, major histocompatibility complex; li, invariant chain; hIi, human Ii; mcIi, cDNA mIi; ER, endoplasmic reticulum; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; C11E9, polyoxyethylene 9 lauryl ether; DSP, diethyldithio-carbonyl; DTSSP, 3,3’dithiobis(sulfo)propionimidyl propionate; sulfo-EGS, ethylene glycol bis(sulfosuccinimidyl succinate); DGS, disuccinimidyl glutarate; PAGE, polyacrylamide gel electrophoresis; aa, amino acid.
8) restriction endonuclease sites, mIIΔ153–215 was generated. For construction of mIIΔ128–215, the starting DNA was cut with restriction endonuclease NsiI (exon 4) and SacII (exon 8). The sequence between these restriction enzyme sites was replaced by a double-stranded oligonucleotide composed of the oligonucleotides 5′-TCGAC-
TAGTTAGGC-3′ and 5′-GGCTAAGTGTTCAA-3′, containing the respective NsiI and SacII restriction sites. The resulting construct has a TGA stop codon in frame, which terminates translation after His127. The mIIΔ162–215 is derived from the starting construct by restriction endonuclease digest with BglII (exon 6), treatment with DNA polymerase I Klenow fragment, and religation. This construct encodes a mutant protein that contains residues 1–162 of mII and, because of a reading frameshift, has an additional unrelated sequence (DLREDEAVL) in positions 163–174, followed by translation termination. All constructs were expressed in the pCEV-3 expression vector.

Antibodies—BU45 is a mouse monoclonal antibody that recognizes a conformation-dependent epitope within the luminal part of human II (31). Purified BU45 was purchased from The Binding Site (San Diego, CA). In1 is a monoclonal rat antibody (IgG2b) recognizing an epitope in the cytosolic tail of mouse II (13). In1 does not bind well to protein A-Sepharose beads, and an additional monoclonal mouse antibody against the constant region of rat antibody κ chain, mouse anti-rat (MAR 18.5) (32), was used for immunoprecipitation. In immunofluorescence microscopy experiments, In1 and MAR 18.5 were used with goat anti-mouse FITC (Zymed Laboratories, San Francisco, CA) as the third antibody.

Cells and Cell Culture—COS cells are Green monkey kidney cells, originating from CV1 cells by stable transfection with an origin-defective mutant of the SV40 vector, coding for the wild-type T antigen (33) originating from CV1 cells by stable transfection with an origin-defective mutant of the SV40 vector (34). COS cells were obtained from ATCC (CCL70). COS cells are reported to be devoid of endogenous mouse Ii (35). A defective mutant of the SV40 vector, coding for the wild-type T antigen (33) originating from CV1 cells by stable transfection with an origin-defective mutant of the SV40 vector (34). COS cells were obtained from ATCC (CCL70). COS cells are reported to be devoid of endogenous mouse Ii (35). A defective mutant of the SV40 vector, coding for the wild-type T antigen (33) originating from CV1 cells by stable transfection with an origin-defective mutant of the SV40 vector (34). COS cells were obtained from ATCC (CCL70). COS cells are reported to be devoid of endogenous mouse Ii (35). A defective mutant of the SV40 vector, coding for the wild-type T antigen (33) originating from CV1 cells by stable transfection with an origin-defective mutant of the SV40 vector (34). COS cells were obtained from ATCC (CCL70). COS cells are reported to be devoid of endogenous mouse Ii (35). A defective mutant of the SV40 vector, coding for the wild-type T antigen (33) originating from CV1 cells by stable transfection with an origin-defective mutant of the SV40 vector (34). COS cells were obtained from ATCC (CCL70). COS cells are reported to be devoid of endogenous mouse Ii (35). A defective mutant of the SV40 vector, coding for the wild-type T antigen (33) originating from CV1 cells by stable transfection with an origin-defective mutant of the SV40 vector (34). COS cells were obtained from ATCC (CCL70). COS cells are reported to be devoid of endogenous mouse Ii (35). A defective mutant of the SV40 vector, coding for the wild-type T antigen (33) originating from CV1 cells by stable transfection with an origin-defective mutant of the SV40 vector (34). COS cells were obtained from ATCC (CCL70). COS cells are reported to be devoid of endogenous mouse Ii (35). A defective mutant of the SV40 vector, coding for the wild-type T antigen (33) originating from CV1 cells by stable transfection with an origin-defective mutant of the SV40 vector (34). COS cells were obtained from ATCC (CCL70). COS cells are reported to be devoid of endogenous mouse Ii (35). A defective mutant of the SV40 vector, coding for the wild-type T antigen (33) originating from CV1 cells by stable transfection with an origin-defective mutant of the SV40 vector (34). COS cells were obtained from ATCC (CCL70). COS cells are reported to be devoid of endogenous mouse Ii (35). A defective mutant of the SV40 vector, coding for the wild-type T antigen (33) originating from CV1 cells by stable transfection with an origin-defective mutant of the SV40 vector (34). COS cells were obtained from ATCC (CCL70). COS cells are reported to be devoid of endogenous mouse Ii (35). A defective mutant of the SV40 vector, coding for the wild-type T antigen (33) originating from CV1 cells by stable transfection with an origin-defective mutant of the SV40 vector (34). COS cells were obtained from ATCC (CCL70). COS cells are reported to be devoid of endogenous mouse Ii (35). A defective mutant of the SV40 vector, coding for the wild-type T antigen (33) originating from CV1 cells by stable transfection with an origin-defective mutant of the SV40 vector (34). COS cells were obtained from ATCC (CCL70). COS cells are reported to be devoid of endogenous mouse Ii (35). A defective mutant of the SV40 vector, coding for the wild-type T antigen (33) originating from CV1 cells by stable transfection with an origin-defective mutant of the SV40 vector (34). COS cells were obtained from ATCC (CCL70). COS cells are reported to be devoid of endogenous mouse Ii (35). A defective mutant of the SV40 vector, coding for the wild-type T antigen (33) originating from CV1 cells by stable transfection with an origin-defective mutant of the SV40 vector (34). COS cells were obtained from ATCC (CCL70). COS cells are reported to be devoid of endogenous mouse Ii (35). A defective mutant of the SV40 vector, coding for the wild-type T antigen (33) originating from CV1 cells by stable transfection with a...
Cross-linking and immunoprecipitation of different hIi constructs. Transiently transfected COS cells were labeled with \([35S]cysteine/methionine\) for 1 h and chased for 30 min. Constructs. Transfected COS cells were labeled with \([35S]cysteine/methionine\) for 1 h and chased for 30 min. Transiently transfected COS cells were labeled with \([35S]cysteine/methionine\) for 1 h and chased for 30 min.

Fig. 2. Cross-linking and immunoprecipitation of different hIi constructs. Transiently transfected COS cells were labeled with \([35S]cysteine/methionine\) for 1 h and chased for 30 min. Lanes 1, 2, 3, 4, and 5 represent COS cells transfected with p35* hIi, p33 hIi, pΔ36 hIi, pΔ50 hIi, pΔ26 hIi, and pΔ30 hIi, respectively, and immunoprecipitated with BU45. A, samples cross-linked with DSP, 6–12% SDS-PAGE, non-reducing conditions. B, samples cross-linked with DSP, 12% SDS-PAGE, reducing conditions. C, samples not cross-linked, 12% SDS-PAGE, non-reducing conditions.

Linked samples. Full-length mli, like hli (Fig. 2), multimerized as a complex of about 90 kDa, corresponding to a trimer (Fig. 4A, lane 1). The mli cDNA (mcli) gave identical results (Fig. 4A, lane 2). Transfection of mli Δ81–127, mli Δ110–130, mli Δ110–161, and mli Δ192–212 resulted in bands corresponding to the molecular weight of trimers (Fig. 4A, lanes 3, 4, 5, and 9), whereas no trimers were visible for mli Δ126–215, mli Δ153–208, mli Δ153–215, and mli Δ128–215 (lanes 6, 7, 8, and 10). Constructs that do not encode exon 6B also trimerize. The exon 6B-encoded sequence is therefore not essential for the self-association of Ii, and we can conclude that the essential sequence for Ii trimerization is contained within residues 153–191. To further investigate the trimerization region, mcli was mutated to encode a stop codon at position 163. This gave a construct encoding aa 1–162 of mli (Fig. 4, A and B, lane 11). Cross-linking of this construct did not produce any trimer, suggesting that the Ii trimerization site lies within residues 163–191, which are encoded by exon 6. The non-trimerizing group of constructs, lacking exon 6, ran as monomers, dimers, and higher molecular weight complexes, probably aggregates. Monomers were also detected for C-terminal deletion constructs that make trimers (except for mli Δ192–212 (Fig. 4A, lane 9)), indicating that trimerization of these deletion constructs was not complete. When the multimers were resolved by reducing the cross-linking agent DSP by dithiothreitol (Fig. 4B), the main bands migrated as expected for monomers.

To summarize, four of the nine deletion constructs make trimers (Fig. 4A, lanes 3, 4, 5, and 9), whereas no trimers were detected for the other constructs. The common denominator for the “trimerization-group” is that all constructs contain exon 6 (residue 162–191), whereas the others do not, strongly indicating that sequences encoded by exon 6 are essential for Ii trimer formation in intact cells.

Intracellular Localization of mli and Deletion Constructs—Ii contains, in its cytosolic tail, signals for sorting to endosomes (16, 17, 19). Furthermore, high expression levels of full-length Ii induce large endosomal structures in a subpopulation of the transfectants (7, 21). The large endosomes are characterized by size and by the fact that the N-terminal 11 amino acids of Ii are essential for their induction (7, 21). To study the influence of the luminal part of Ii on its intracellular distribution, immunofluorescence microscopy studies were performed on COS and CV1 cells transfected with mli and mli C-terminal deletion constructs. All the resulting proteins were located in vesicular structures (indicated by long and short arrows in Fig. 5). Cells transfected with constructs that contain exon 6 showed, in addition, large endosomal structures (Fig. 5, long arrows). To assure that the large vesicles had the same characteristics as those described earlier (7, 21), we allowed some of the transfected cells to internalize albumin-Texas Red for 1 h at 37 °C.
an increased volume and contain more material, and probably also fluid phase marker, than smaller endosomes. It is therefore not possible to compare the different sized compartments since the material maintained in the small endosomes may be below detection level. The detectable level of fluid phase marker co-localizing with Ii in the large endosomes after 6 h do, however, suggest that the fluid phase marker is retained, as endocytosed material normally reaches the lysosomes after 1–3 h (38, 39). As no constructs lacking exon 6 induced changes in endosomal morphology, our results thus suggest that the exon 6-encoded sequence is a prerequisite for the formation of large endosomal structures.

**DISCUSSION**

The ability of Ii to make trimers in a cell-free system has been assigned to residues 163–183 (37). An earlier hypothesis of Elliot et al. (40) suggested involvement of a predicted amphipathic helix, between residues 146–164 in Ii self association. NMR studies performed by Jasanoff et al. (41) show that a luminal trimeric part of the invariant chain, expressed in E. coli, contains a protease resistant, compact, and well structured region (aa 118–193). This indicates that the luminal region involved in trimerization might go beyond aa 163–183, even though these aa are essential for initiation of the trimer formation. In our study, we have expressed various cytosolic and luminal deletion mutants in COS cells and found that the Ii luminal segment encoded by exon 6 (residues 162–191) is essential for the formation of Ii trimers. The cytosolic tail and all the luminal domains of Ii, except the sequence encoded by exon 6, were not essential for trimer formation although the fraction of detected trimers was lower for some of the deletions. This shows that trimerization is not induced by the N-terminal cytosolic tail although NMR studies have shown that a peptide representing the tail is found as a homotrimer. Various chemical cross-linkers were generally needed to stabilize the multimers, but trimers of p35* hIi, p33 hIi, and Δ20 hIi were also detected under nonreducing conditions without the use of cross-linker. This might indicate that parts of the cytosolic tail of Ii have a stabilizing effect on the trimer after its induction. This is supported by the work by Amigorena et al. (42) where they showed that a leupeptin-induced invariant chain peptide of 10 kDa was still in a trimeric conformation even though it only contained aa 1–98.

From our data and those of Bijlmakers et al. (37), we have concluded that the region responsible for the formation of trimers is located at exon 6. The study of Bijlmakers et al. (37) was limited to the *in vitro* ER events since microsomes were used to study the interactions, whereas we analyzed the trimerization of Ii in transfected cells. The study of Newcomb and Cresswell (31) showed that the trimeric complex was maintained throughout the biosynthetic pathway of Ii, most likely including endosomes. Degradation of Ii occurs from the C terminus, and by inhibiting degradation by adding leupeptin, they got an Ii-degradation product of approximately 140 amino acids, called leupeptin-induced protein (LIP). The nonamer (MHC class II α- and β-chain-LIP) is no longer kept together under conditions where the full size (αβII)3 complex is maintained, hence the (αβII)3 complex is disturbed upon partial Ii degradation. This suggests that Ii remains in a trimeric form at least until residues encoded by exon 6 are degraded and maybe even longer (42). Our data show that the full-length Ii, without the MHC class II, are found to be exclusively trimeric after a 1-h labeling and 30-min chase. At this time point, a significant fraction is expected to have passed the Golgi apparatus, imply-

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2 A. Motta, P. Amodeo, P. Fucile, M. A. Castiglione Morelli, B. Bremnes, and O. Bakke, submitted for publication.
ing that Ii remains trimeric until it reaches the endosomal pathway.

The more detailed sequence requirements for the trimerization of Ii are not known. Residues encoded by exon 6, which we find to be the essential region for trimerization of Ii, are 70% identical in mouse, rat, and human, whereas with chicken, the identity is about 30% (Fig. 7). In the sequence of human, mouse, rat, and chicken Ii, there are three well conserved sequences, one in the N-terminal tail, one in the transmembrane region, and the last in exon 6. The conserved region encoded by exon 6 is a largely unpolar sequence, Phe-Glu-Ser-Thr-Trp-Met-His-Lys-Trp-Leu-Leu-Phe-Glu-Met, and this stretch of residues is thus a potential candidate for the core sequence required for the initiation of the Ii homotrimer formation.

Ii is detected in endosomal vesicles in antigen-presenting cells as well as in transfected cells, with or without MHC class II molecules (16–18, 43). The transfected mouse Ii deletion constructs with an intact cytosolic tail were all transported to endosomal vesicles, either by direct transport or by internalization from the plasma membrane. Ii with exon 6 deleted exists mostly in a monomeric form but is still sorted to endosomes. The chimeric protein INA (a tetrameric protein) containing the Ii cytosolic tail fused to the transmembrane and luminal domains of neuraminidase (NA), is also sorted to endosomes (19). We may therefore conclude that trimerization of Ii is not essential for endosomal sorting. Such a conclusion is supported by the data of Arneson and Miller (44), showing that Ii complexes with monomeric tails also mediate transport to the endosomal pathway.

Romagnoli et al. (21) showed that high expression levels of Ii induced large endosomal structures in a subpopulation of transfected COS cells. Cells with these enlarged endosomal vesicles retained fluid phase markers in the large endosomes for a prolonged period of time before reaching the lysosomes. Large vesicular structures were also found in the human fibroblast M1 cell line (22, 45) and canine Madin-Darby canine kidney cells, stably transfected with Ii or Ii and MHC class II. Although large endosomal structures are not seen in most

\[3\] A. Simonsen, E. Stang, B. Bremnes, M. Røe, K. Prydz, and O. Bakke, submitted for publication.
FIG. 6. Delay of endocytosis in cells transfected with constructs containing exon 6. COS cells transiently transfected with mci (A and B), mli Δ81–127 (C and D), and mli Δ110–130 (E and F) were pulsed with albumin-Texas Red (1 mg/ml) for 1 h and chased for 1 or 6 h (mli Δ81–127 (G and H)). The cells were then labeled with antibody (In1, MAR 18.5, and FITC-labeled goat anti-mouse). A, C, E, and G show the labeling for mli with FITC, and B, D, F, and H show the labeling for mci with FITC, and B, D, F, and H show the remains of Texas Red labeling. Bar = 20 μm for all constructs.

The lack of Ii in the native antigen presenting cells, they are observed in Langerhans cells (46). We might speculate that the phenotypic changes induced by Ii and the delay of anterograde endosomal transport of fluid phase markers might be advantageous for antigen presentation, as MHC class II molecules and antigen could co-reside in the endosomes for a prolonged period of time. Studies performed on cells expressing these enlarged endosomal structures show that these organelles may be considered as both early endosomes and late endosomes (7, 21, 45), but except for the delay in anterograde transport of fluid phase markers, they are functionally similar to normal endosomes. Ii lacking the first 11 amino acids does not induce large vesicular structures (7, 21). Further elucidation of the signal mediated by the first 11 amino acids has revealed that N-terminal acidic charges and the first di-leucine based signal in Ii were both essential for the induction of the large endosomes (47). However, as shown here, such signals may induce large vesicular structures only in combination with a trimeric Ii. The study by Pond et al. (47) showed that N-terminal acidic residues and di-leucine-based internalization signals from other proteins can mediate the induction of large endosomal structures when transferred to Ii but not in their original surroundings. This and our earlier results with Ii fusion proteins (7, 19) indicate that there is more than one requirement in Ii that has to be

fulfilled to induce enlargement of the endosomes.

Ii is associated with MHC class II molecules, and the trimer of Ii is a prerequisite for the formation of the transport competent (αβli)_3 nonameric complex (4). Ii, or more specifically trimeric Ii, is probably necessary for the correct folding of MHC class II molecules as MHC class II molecules expressed in cells lacking Ii have an altered conformation (48). For human Ii, it has been suggested that the trimer of Ii is necessary to retain the α, β and Ii molecules in the ER to allow the correct functional conformation to occur (4). Another theory, presented by Roche et al. (5), proposed that the trimer is necessary to prevent the binding of peptides to MHC class II molecules in the ER. Arneson and Miller (44), showed that the localization signals encoded by residues 2–17 in p33 hIi must be in a dimeric or trimeric form to mediate a direct transport to the endosomal pathway. This may also be an important function of the trimer. Recent results of Bertolino et al. (49) indicate that the function of trimerization is of importance for antigen presentation, as the region of Ii essential for the presentation of some peptides from HEL (hen egg lysozyme), residues 131–191, also covers the region of Ii essential for multimerization. These theories for the function of trimeric Ii may all be true; however, since the mechanisms involved are not known, we may not yet fully understand the significance of the trimerization of Ii for the endosomal transport process or for antigen presentation.

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