A Short Basic Domain Supports a Nucleic Acid-Binding Activity in the Rice Tungro Bacilliform Virus Open Reading Frame 2 Product

E. Jacquot, M. Keller, and P. Yot

Institut de Biologie Moléculaire des Plantes du Centre National de la Recherche Scientifique, Université Louis Pasteur, 12 rue du Général Zimmer, 67084 Strasbourg Cedex, France

Received August 11, 1997; returned to author for revision September 3, 1997; accepted September 24, 1997

Little is known about the features of badnavirus open reading frame 2 products (P2). So far, no consensus functional domain has been found in these proteins. However, they all have in common at their C-terminus amino acids which may have the capacity to bind nucleic acids. Such capacity has already been established for cacao swollen shoot virus protein P2. We have looked for such a binding capacity of rice tungro bacilliform virus (RTBV) ORF 2 product. For this purpose, the protein was expressed as full-length or truncated versions in Escherichia coli. When used in nucleic acid-binding assays, complete RTBV P2 was shown to bind both DNA and RNA. This property may be related to a basic sequence, PPKG/KRKYP, localized at its C-terminus. Mutations were introduced into this sequence and revealed that four of the five basic residues, including a crucial lysine, are required for the binding to nucleic acids. Moreover, this sequence can confer binding capacity when it is fused to the N-terminus of nonbinding proteins.

Rice tungro bacilliform virus (RTBV) belongs to the badnavirus subgroup (Lockhart, 1990) of plant pararetroviruses (for a review, see Rothnie et al., 1994), the type member being commelina yellow mottle virus (CoYMV) (Medberry et al., 1990). Rice tungro disease is present in countries of south and southeast Asia (Hibino, 1987) and is responsible for reduced yield and crop losses estimated at $1.5 billion (Herdt, 1991). It is caused by the complex of RTBV and rice tungro spherical virus, RTSV, an RNA virus which is transmitted independently by a leafhopper vector (Nephotettix virescens) (Hibino et al., 1978; Jones et al., 1991). The nonenveloped bacilliform RTBV particle (35 nm in diameter and 150–350 nm in length) encloses a circular double-stranded DNA molecule of about 8.0 kbp (Qu et al., 1991; Hay et al., 1991). The genome is interrupted by two site-specific discontinuities (Jones et al., 1991), one on each strand, resulting from its replication by reverse transcription (Laco and Beachy, 1994) as for caulimoviruses, the second plant pararetrovirus subgroup (type member cauliflower mosaic virus, CaMV). Four putative functional open reading frames (ORF) coding for proteins larger than 10 kDa are located on the plus strand of RTBV genome (Fig. 1). The ORF 1, 2, and 3 products are synthesized from the more-than-genome length RNA, which is also used as substrate for genome amplification by reverse transcription (for a review, see Hull, 1996), whereas the ORF 4 product is believed to result from a spliced form of this transcript (Füttener et al., 1994). Synthesis of ORF I protein (24 kDa) is initiated at an AUU codon (Füttener et al., 1996). The function of this protein as well as of the ORF 4 product (46 kDa) is not yet known. ORF 3 codes for a polypeptide of 194 kDa (P3). Sequence comparisons between plant pararetrovirus proteins (Franck et al., 1980; Richins et al., 1987; Hull et al., 1986; Medberry et al., 1990; Qu et al., 1991; Hagen et al., 1993) suggest that P3 contains (from N- to C-terminus) a movement protein domain, the coat protein, a consensus sequence for aspartyl proteinase, reverse transcriptase, and RNase H. ORF 2, located between nucleotides 665 and 997 (Qu et al., 1991), encodes a 12-kDa protein (P2) of 110 amino acids. Few data concerning the properties and the putative function of this protein and of other badnavirus P2 proteins are available.

P2 was shown to be associated with CoYMV or RTBV particles (Cheng et al., 1996; Hull, 1996) and cacao swollen shoot virus (CSSV) P2 protein was described as a sequence nonspecific nucleic acid-binding protein (Jacquot et al., 1996). Badnavirus P2 proteins all have basic and hydrophobic residues and prolines at their C-terminus. Such amino acids are also present at the C-termini of caulimovirus ORF III products (PIII) and of bacterial histone-like proteins (Mougeot, 1995). The C-termini of both CSSV P2 and CaMV PIII proteins support nucleic acid binding. Based on these observations, these two proteins were assumed to have similar function(s) in their
NCLEIC ACID-BINDING ACTIVITY IN RTBV

respective viral cycle (Jacquot et al., 1996). Here we report binding assays carried out with RTBV P2 protein in order to determine whether this protein binds DNA and RNA as does CSSV P2, to further characterize the amino acid motif involved in the putative binding property, and to study the possible use of such a binding domain as a new tag in protein technology.

MATERIALS AND METHODS

Plasmid constructions

The full-length RTBV genome cloned in pBluescript (Stratagene) (Qu et al., 1991) served as template for PCR amplification of ORF 2. Escherichia coli DH5α (Stratagene) and BL21/DE3(pLysS) (Studier and Moffatt, 1986) strains were used as hosts for plasmid DNA preparation and protein expression, respectively. PCR was performed using the oligonucleotide primer pair fp0 as forward primer and rp0 as reverse primer (Table 1). The amplified fragment was cloned into the NdeI and BamHI sites of the procaryotic expression vector pET-3a in front of the T7 RNA polymerase promoter (Rosenberg et al., 1987). The resulting plasmid pRT2 allows synthesis of the complete RTBV P2 protein. Similarly, plasmids were constructed in order to produce a set of P2 proteins truncated or mutated at the C-terminus using the above forward primer and one of the reverse primers mentioned in Table 1. The amplified plasmids were designated pRTΔ2, pRTΔ12, pRTKRA, pRTKAK, pRTARK, pRTAAK, pRT(Δ)3, pRT(Δ)2, and pRT(Δ)5, respectively (Table 1). The pET-3a-derived plasmid pRT2BD was used to produce CSSV deoxy P2 protein deleted of 31 amino acids at its C-terminus. Plasmid encoding CaMV PIII truncated of 18 amino acids at the C-terminus was generated using the appropriate primer pair and CaMV DNA purified from virions as substrate for PCR; it was designated pETCa3Δ18 and allowed synthesis of CaMV PIIIΔ18. Finally, constructions were performed in view of obtaining chimeric proteins containing the C-terminus of RTBV P2. PCR was carried out with the oligonucleotide primer pair ct+ as forward primer and ct− as reverse primer (Table 2) to amplify a fragment corresponding to the 3′ sequence of RTBV ORF 2 (nt 959 to 994). The resulting fragment was cloned into the NdeI and BamHI sites of pET-3a, forming the pLink-3a vector. DNA fragments corresponding to 3′ truncated versions of CaMV ORF III, CSSV ORF 2, or RTBV ORF 2 flanked by KpnI and CeiI sites were produced by PCR, using the appropriate primer pairs (Ca+ and Ca−, CS+ and CS−, or RT+ and RT−) (Table 2) and DNA substrate (genomic DNA of either CaMV, CSSV, or RTBV, respectively). PCR products were cloned into KpnI and CeiI restriction sites of pLink-3a. The resulting plasmids were designated pETCa3Δ, pCS2BDΔ, and pRT2BDΔ and allowed expression of proteins pCa3BD, pCS2BD, and pRT2BD, respectively. All the plasmid constructions were confirmed to be error-free by sequencing using the diodexy chain termination method.

Expression of complete RTBV P2 protein and mutated versions

Recombinant E. coli BL21/DE3(pLysS) was grown and induced as described (Jacquot et al., 1996). After induction for 2 h, bacteria were lysed under pressure in a French press (9000 p.s.i.) and lysate was centrifuged (12,000 g for 10 min), yielding the supernatant S1. After heat treatment of the latter at 65°C for 15 min and centrifugation as above, the supernatant S2 was obtained. Protein concentration was determined according to Bradford (1976). Proteins were separated by 18% sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and visualized after staining with Coomassie blue.

Production of antisera and Western blot analysis

The complete RTBV P2 protein was used to immunize rabbits and raise antisera as follows. Proteins present in lysate of induced bacteria were separated by 18% SDS-PAGE and stained with Coomassie blue. Gel bands
### TABLE 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Protein encoded by PCR-derived products</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Forward primer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fp0</td>
<td>5'agtaagtgcc DATGACGCTGATTATCCTAACTTTCAAGG3'</td>
<td>P2</td>
</tr>
<tr>
<td><strong>Reverse primer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rp0</td>
<td>5'gccgcagagtcTCA TCTTCACTGATTATCCTAACTTTCAAGG3'</td>
<td>P2</td>
</tr>
<tr>
<td>rpΔ2</td>
<td>5'gccgcagagtcTCAATTTTTTCTTTATTTGATTATCCTAACTTTCAAGG3'</td>
<td>P2Δ2</td>
</tr>
<tr>
<td>rpΔ12</td>
<td>5'gccgcagagtcTCAATTTTTTCTTTATTTGATTATCCTAACTTTCAAGG3'</td>
<td>P2Δ12</td>
</tr>
<tr>
<td>rpmKRA</td>
<td>5'gccgcagagtcTCA TCTTCACTGATTATCCTAACTTTCAAGG3'</td>
<td>P2KRA</td>
</tr>
<tr>
<td>rpmKAK</td>
<td>5'gccgcagagtcTCA TCTTCACTGATTATCCTAACTTTCAAGG3'</td>
<td>P2KAK</td>
</tr>
<tr>
<td>rpmARA</td>
<td>5'gccgcagagtcTCA TCTTCACTGATTATCCTAACTTTCAAGG3'</td>
<td>P2ARA</td>
</tr>
<tr>
<td>rpmAAK</td>
<td>5'gccgcagagtcTCA TCTTCACTGATTATCCTAACTTTCAAGG3'</td>
<td>P2AAK</td>
</tr>
<tr>
<td>rpm(A)</td>
<td>5'gccgcagagtcTCA TCTTCACTGATTATCCTAACTTTCAAGG3'</td>
<td>P2(A)</td>
</tr>
<tr>
<td>rpmKRA</td>
<td>5'gccgcagagtcTCA TCTTCACTGATTATCCTAACTTTCAAGG3'</td>
<td>P2KRA</td>
</tr>
<tr>
<td>rpmKAK</td>
<td>5'gccgcagagtcTCA TCTTCACTGATTATCCTAACTTTCAAGG3'</td>
<td>P2KAK</td>
</tr>
<tr>
<td>rpmARA</td>
<td>5'gccgcagagtcTCA TCTTCACTGATTATCCTAACTTTCAAGG3'</td>
<td>P2ARA</td>
</tr>
<tr>
<td>rpmAAK</td>
<td>5'gccgcagagtcTCA TCTTCACTGATTATCCTAACTTTCAAGG3'</td>
<td>P2AAK</td>
</tr>
<tr>
<td>rpm(A)</td>
<td>5'gccgcagagtcTCA TCTTCACTGATTATCCTAACTTTCAAGG3'</td>
<td>P2(A)</td>
</tr>
<tr>
<td>rpmKRA</td>
<td>5'gccgcagagtcTCA TCTTCACTGATTATCCTAACTTTCAAGG3'</td>
<td>P2KRA</td>
</tr>
<tr>
<td>rpmKAK</td>
<td>5'gccgcagagtcTCA TCTTCACTGATTATCCTAACTTTCAAGG3'</td>
<td>P2KAK</td>
</tr>
<tr>
<td>rpmARA</td>
<td>5'gccgcagagtcTCA TCTTCACTGATTATCCTAACTTTCAAGG3'</td>
<td>P2ARA</td>
</tr>
<tr>
<td>rpmAAK</td>
<td>5'gccgcagagtcTCA TCTTCACTGATTATCCTAACTTTCAAGG3'</td>
<td>P2AAK</td>
</tr>
<tr>
<td>rpm(A)</td>
<td>5'gccgcagagtcTCA TCTTCACTGATTATCCTAACTTTCAAGG3'</td>
<td>P2(A)</td>
</tr>
</tbody>
</table>

a Oligonucleotide primers include restriction sites underlined and corresponding to Ndel and BanHI for forward and reverse primers, respectively. Nucleotides complementary to the viral sequences are in bold uppercase letters, mutated nucleotide sequence within the viral sequence are in bold lowercase letters, and nonviral nucleotide sequences are in normal lowercase letters.

b Mutated amino acids are underlined.

containing approximately 80 μg of P2 were excised and crushed in PBS buffer (v/v). The crushed gel was then emulsified with an equal volume of complete Freund's adjuvant and divided into two fractions before being subcutaneously injected into two rabbits. Crude sera were used at 1/10,000 for Western blot analysis. After SDS–PAGE and electrotransfer onto nitrocellulose membrane, immunogenic proteins were detected using alkaline phosphatase goat anti-rabbit IgG (Sigma) and visualized in the presence of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Nucleic acid-binding assays

Nucleic acid-binding assays were performed as previously described (Jacquot et al., 1996). Probes were RTBV genome [32P]DNA obtained by random priming (Feinberg and Vogelstein, 1983) or [32P]RNA made by in vitro transcription (Greif et al., 1990) of a RTBV DNA fragment (nt 665–997). Probes corresponding to CaMV genome [32P]DNA or [32P]RNA transcript of a CSSV DNA fragment (nt 869–1267) were also used.

RESULTS

Expression of complete and truncated RTBV P2 proteins and nucleic acid-binding assays

The procatyotic expression system consisting of a pET-3a-derived vector allows synthesis of proteins without fusion domains. After lysis and heat treatment of the induced recombinant bacteria BL21/DE3(pLysS, pRT2), analysis by 18% SDS–PAGE revealed that RTBV P2 protein was present in supernatant S2 (Fig. 2A, lane 1). Densitometry of the gel shows that P2 represents 65% of the S2 proteins. Recombinant bacteria transformed with truncated RTBV ORF 2 plasmids were also induced in order to produce P2 proteins deleted of either 2 or 12 residues from the C-terminus. The corresponding proteins P2Δ2 and P2Δ12 were produced in amounts equivalent to that of the complete protein and were also found in the soluble fraction S2 (Fig. 2A, lanes 2 and 3). After SDS–PAGE and electrotransfer onto nitrocellulose membrane, proteins were renatured and incubated with either RTBV RNA (Fig. 2B) or RTBV DNA (Fig. 2C) labeled with [32P]. Under our experimental conditions, none of the bac-
TABLE 2

Oligonucleotides Used as Primers to Generate PCR Products for the Construction of pLink-3a and Its Derived-Plasmids

<table>
<thead>
<tr>
<th>DNA substrate for PCR</th>
<th>Primer Name</th>
<th>Sequence*</th>
<th>Nucleotide coordinatesb</th>
<th>Region of the viral protein corresponding to PCR productc</th>
</tr>
</thead>
<tbody>
<tr>
<td>RTBV ORF II</td>
<td>ct+</td>
<td>5′-agtggccatATG-3′</td>
<td>959–989 (+)</td>
<td>99–110</td>
</tr>
<tr>
<td>CaMV ORF III</td>
<td>Ca+</td>
<td>5′-agtggccatATG-3′</td>
<td>993–110 (+)</td>
<td>2–111</td>
</tr>
<tr>
<td>CSSV ORF II</td>
<td>CS+</td>
<td>5′-agtggccatATG-3′</td>
<td>987–110 (+)</td>
<td>2–101</td>
</tr>
<tr>
<td>RTBV ORF II</td>
<td>RT+</td>
<td>5′-agtggccatATG-3′</td>
<td>981–110 (+)</td>
<td>2–98</td>
</tr>
</tbody>
</table>

*a Oligonucleotide primers include underlined restriction sites. Nucleotides complementary to the viral sequences are in bold uppercase letters and nonviral nucleotide sequences are in normal lowercase letters. Start and stop codons are denoted by *.

*b Nucleotide coordinates according to Qu et al. (1991), Franck et al. (1980), and Hagen et al. (1993) for RTBV, CaMV, and CSSV, respectively. (+) and (−) correspond to plus and minus DNA strands, respectively.

c Full size of RTBV ORF 2, CaMV ORF III, and CSSV ORF 2 products are 110, 129, and 131 amino acids, respectively.

terial proteins bound nucleic acids (Figs. 2B and 2C, lanes 4), whereas P2 interacted with both DNA and RNA (lanes 1). RTBV P2 protein also bound heterologous nucleic acids (CaMV DNA, pBR322 DNA, or CSSV RNA) (not shown). A truncated version of P2Δ2 was able to bind both types of nucleic acid at levels similar (Figs. 2B and 2C, lanes 2) to those observed with complete RTBV P2 protein, whereas deletion of the last 12 amino acids prevented binding (Figs. 2B and 2C, lanes 3). Consequently, the 12 residues 99PPKKGIKRKYPA110 were assumed to constitute the binding domain (BD).

Effect of point mutations in the C-terminus of the P2 protein on its nucleic acid-binding capacity

Point mutated P2 proteins were produced by substitution in BD of alanine for a basic residue (Table 1) and each was tested for nucleic acid-binding ability. First, a blotted membrane was analyzed with antiserum raised against RTBV P2 to verify that equivalent amounts of the different mutated P2 proteins were fixed on the membrane (Fig. 3A). P2 proteins containing only one point mutation were as efficient in binding to RNA or DNA (Figs. 3B and 3C, lanes 2, 6, 8, and 10) as P2Δ2 protein (lane 12) except for mutant P2ARK (lanes 4), which weakly binds DNA and RNA. Binding patterns indicate that among mutants in which more than one basic residue was replaced by alanine, P2(A)5, P2(A)2, and P2(A)3 no longer have binding capacity (Figs. 3B and 3C, lanes 5, 9, and 13), whereas P2KAA (lane 1), P2AAK (lane 3), and P2ARA (lane 7) bind DNA slightly.

Property of chimeric proteins containing the RTBV P2 binding domain

Insertion of the 3′ end of RTBV ORF 2 corresponding to BD into NdeI and BamHI sites of pET-3a leads to the pLink-3a expression vector (Fig. 4). The latter allows expression of fusion proteins containing the last 12 amino acids of RTBV P2 protein at their N-terminus. CaMV PIII, RTBV P2, and CSSV P2 proteins deleted of 18, 12, and 31 C-terminal amino acids, respectively, as well as the corresponding proteins fused to BD, were expressed in bacteria. SDS PAGE analysis of S1 fractions is illustrated in Fig. 5A. All the different overexpressed proteins are soluble in bacterial lysate and present in almost equivalent amounts. Lanes 1, 4, and 6 correspond to truncated CaMV PIII, RTBV P2, and CSSV P2 proteins, respectively. These proteins do not bind nucleic acids (Figs. 5B and 5C, lanes 1, 4, and 6) as already
amino acids were shown to be involved in nucleic acid-binding in vitro (Jacquot et al., 1996). In this report, we looked for the same feature in RTBV P2. The corresponding ORF 2 was cloned into a procaryotic expression vector. RTBV P2 was expressed in E. coli and mainly found, as was the CSSV P2 protein, in the soluble fraction of the bacterial lysate after heat treatment. When used in nucleic acid-binding assays, it also interacted with DNA and RNA in a sequence-nonspecific manner. Assays carried out with C-terminal truncated versions of RTBV P2 showed that a very short sequence 99PPKKGIKRKYPA110 supports this property. The relatively small size of this binding region, compared with that of CSSV P2 (20 C-terminal amino acids), and the presence of five basic residues which could be directly involved in ionic interactions with nucleic acids prompted us to investigate the effects of mutations in this sequence. The importance of basic amino acids in the binding was determined with P2(A)5 protein in which the five basic residues were replaced by alanines. This protein is no longer able to bind DNA and RNA. The basic domain can be arbitrarily divided in the two subdomains KK and KRK which are both required for nucleic acid-binding activity. Indeed, P2Δ6 protein with 99PPKKGI104 as C-terminal sequence (not shown), and P2(A)1 and P2(A)2 proteins in which KRK and KK are respectively replaced by alanine residues, do not bind nucleic acids. Subsequently, the role of each basic residue was studied in the two subdomains. Substitution of only one alanine for lysine or arginine in each subdomain does not affect the binding capacity of the P2 protein except for K105, which cannot be changed since P2ARK is inactive in binding. Moreover, proteins P2ARA, P2AAK, and P2KAA with two replaced residues in the KRK subdomain do not bind nucleic acids. This result for the first two mutants is explained by the absence of K105. It indicates that for P2KAA the presence of at least four of the five basic residues, including amino acid K105, is required for DNA- and RNA-protein interaction.

In the present work, interaction between the RTBV P2 and RNA or DNA was shown to occur only through the decapeptide 99PPKKGIKRKY108 since the C-terminal proline and alanine of BD sequence are dispensable. None of the known RNA binding motifs (Burd and Dreyfuss, 1994) can be identified within this decapeptide. The fact that RTBV P2 is also a sequence-nonspecific DNA binding protein which probably contacts the DNA in the minor groove suggests that this protein recognizes the RNA major groove. Indeed, Chen and Frankel (1995) proposed that the major groove of RNA and the minor groove of DNA share common recognition features. Proteins which bind to DNA in the minor groove have been classified by Churchill and Travers (1991) in two major groups, containing proline-rich and α-helical motifs, respectively. Viral sequence-nonspecific DNA binding proteins such as hepatitis B virus coat protein, λ phage 434 repressor, P2 bacteriophage M protein, CaMV PIII, and CSSV P2 proteins are not (data not shown). This result demonstrates the immunogenicity of the RTBV P2 binding domain.

**DISCUSSION**

Badnavirus P2 proteins have no sequence homologies but possess similar residues (basic, hydrophobic, and prolines) at their C-termini. Such residues could confer common properties on the P2 proteins. For CSSV P2, these
FIG. 3. Immunodetection and nucleic acid-binding assays for mutated RTBV ORF 2 products. Blots were probed with either antiserum raised against P2 protein (A), $^{32}$P-labeled single-stranded RNA from CSSV (B), or $^{32}$P-labeled double-stranded DNA from RTBV (C). Lanes contain heat-treated soluble fractions enriched in P2KAA (lanes 1), P2KAK (lanes 2), P2AAK (lanes 3), P2ARK (lanes 4), P2(A) (lanes 5), P2KRA (lanes 6), P2ARA (lanes 7), P2AK (lanes 8), P2(A) (lanes 9), P2KA (lanes 10), P2(R) (lanes 11), P2(R) (lanes 12), or P2(A) (lanes 13). Resulting RNA- and DNA-protein complexes were visualized by autoradiography (B and C).

FIG. 4. Schematic representation of the pET-3a-derived pLink-3a vector. The RTBV ORF 2 nucleotide sequence and corresponding amino acid sequence of the P2 binding domain (BD) are listed in normal and bold uppercase letters, respectively. Unique restriction sites NdeI, KpnI, SacI, BamHI, and CelII surrounding BD are shown by overlining the corresponding sequences. P and T boxes represent the T7 promoter and $\phi$10 terminator, respectively. The black box corresponds to the $\beta$ lactamase gene and the black dot to the procaryotic replication origin.
organization can be extended to the BD motif (Fig. 6B) and allows us to understand the relationship between KK and KRK subdomains which are both essential for the binding activity.

Both RTBV P2 and CSSV P2 are able to bind nucleic acids but they do not share the same binding motif at their C-terminus. Indeed, RTBV P2 belongs to the α-helical group with a decapeptide motif, whereas CSSV P2 is classified in the proline-rich group with a KPK motif (Jacquot et al., 1996). This observation could reflect the result of convergent rather than divergent evolution in the badnavirus group.

The prokaryotic expression vector pLink-3a was constructed to fuse the BD motif to proteins devoid of binding capacity. The resulting fusion proteins were shown to be able to bind nucleic acids and to be detectable with antibodies directed against RTBV P2. The autonomous binding capacity of BD can be explained by the presence at its borders of proline residues which induce bent or kinked structures and which usually fit well in the first three N-terminal residues of α-helixes. In view of these results, the RTBV P2 BD could represent a new tag domain for purification of BD fusion proteins by affinity chromatography with either DNA matrix or IgG-coated matrix, or for detection of proteins in far-western assays (Himmelbach et al., 1996) with BD probes as targets for labeled nucleic acids or antibodies against RTBV P2.

ACKNOWLEDGMENTS

We thank Claude M. Fauquet for providing the cloned RTBV DNA. We are particularly grateful to Philippe Hammann for his help in automatic sequencing of the plasmid constructions. We also thank Kenneth E. Richards for critically reading the manuscript.

REFERENCES


