Agonist-dependent Phosphorylation of the G Protein-coupled Receptor Kinase 2 (GRK2) by Src Tyrosine Kinase*

(Received for publication, May 24, 1999, and in revised form, September 1, 1999)

Susana Sarnago, Ana Elorza, and Federico Mayor, Jr.‡

From the Departamento de Biología Molecular, Centro de Biología Molecular "Severo Ochoa" (Consejo Superior de Investigaciones Científicas-Universidad Autónoma de Madrid) Universidad Autónoma, E-28049 Madrid, Spain

GRK2 is a member of the G protein-coupled receptor kinase (GRK) family, which phosphorylates the activated form of a variety of G protein-coupled receptors (GPCR) and plays an important role in GPCR modulation. It has been recently reported that stimulation of the mitogen-activated protein kinase cascade by GPCRs involves tyrosine phosphorylation of docking proteins mediated by members of the Src tyrosine kinase family. In this report, we have investigated the possible role of c-Src in modulating GRK2 function. We demonstrate that c-Src can directly phosphorylate GRK2 on tyrosine residues, as shown by in vitro experiments with purified proteins. The phosphorylation reaction exhibits an apparent Km for GRK2 of 12 nM, thus suggesting a physiological relevance in living cells. Consistently, overexpression of the constitutively active c-Src Y527F mutant in COS-7 cells leads to tyrosine phosphorylation of co-expressed GRK2. In addition, GRK2 can be detected in phosphotyrosine immunoprecipitates from HEK-293 cells transiently transfected with this Src mutant. Interestingly, phosphotyrosine immunoblots reveal a rapid and transient increase in GRK2 phosphorylation upon agonist stimulation of β2-adrenergic receptors co-transfected with GRK2 and wild type c-Src in COS-7 cells. This tyrosine phosphorylation is maximal within 5 min of isoproterenol stimulation and reaches values of ~5-fold over basal conditions. Furthermore, GRK2 phosphorylation on tyrosine residues promotes an increased kinase activity toward its substrates. Our results suggest that GRK2 phosphorylation by c-Src is inherent to GPCR activation and put forward a new mechanism for the regulation of GPCR signaling.

Activation of G protein-coupled receptors (GPCRs)3 triggers a process termed desensitization, initiated by agonist-dependent phosphorylation of the receptor by specific G protein-coupled receptor kinases (GRKs) (1). This phosphorylation event leads to the recruitment of cytosolic proteins known as β-arrestins to the receptor-signaling complex, with the subsequent uncoupling from heterotrimeric G proteins and loss of receptor responsiveness (1–3). GRK2 is a ubiquitous member of the GRK family that has been shown to phosphorylate different GPCRs (4, 5). Recent evidence indicates that GRK2 and β-arrestin not only promote receptor uncoupling but also directly participate in GPCR sequestration, thus triggering receptor dephosphorylation and recycling to the plasma membrane (reviewed in Refs. 6–8). On the other hand, the recently unveiled role of GRK2 in the phosphorylation of other nonreceptor substrates such as tubulin (9, 10) further indicates the relevance of this kinase in GPCR-mediated signaling.

Consistent with an important physiological role, GRK2 activity and subcellular localization appear to be subject to complex regulatory processes that involve the interaction of diverse domains of the kinase with G protein βγ subunits, several lipids, anchoring proteins, and the agonist-bound form of receptors. The interaction of GRK2 with βγ subunits through its C-terminal pleckstrin homology domain helps to target the kinase to the membrane and enhances its activity toward the receptor (11, 12). The interaction of GRK2 with phosphatidylinositol 4,5-bisphosphate by means of the N-terminal portion of its pleckstrin homology domain seems also to be necessary for full kinase activation (13, 14). GRK2 activity toward receptors is also modulated by phosphorylation by PKC on a yet unidentified residue located in the C terminus of GRK2. Phosphorylation by PKC leads to an increased GRK2 activity, probably due to an enhanced kinase association to the plasma membrane (15–17). This would provide a cross-talk or feedback mechanism by which activation of PKC by different transduction systems may affect GPCR modulation and signaling.

In this regard, emerging evidence indicates that phosphorylation of Gq-or Gi-coupled GPCR also leads to the activation of the mitogen-activated protein kinase (MAPK) cascade in a Ras-dependent way (see Refs. 7 and 18 for recent reviews). It has been shown that activation of lysophosphatidic acid and β2-adrenergic and other GPCR receptors stimulate phosphorylation on tyrosine residues of Shc and Gab1 adapter proteins and other plasma membrane-associated proteins (Refs. 18–21 and references therein). Recruitment and activation of cytosolic tyrosine kinases of the Src family play a critical role in the GPCR-mediated regulation of the MAPK cascade (Refs. 18 and 21–23 and references therein). Interestingly, it has been recently suggested that GRK2 and β-arrestin directly participate in the process of MAPK cascade activation, either by facilitating receptor internalization and/or the interaction with additional proteins (7, 24, 25). On the other hand, overexpression of the c-Src kinase enhances β-adrenergic-induced cAMP accumulation in murine fibroblasts (26, 27) and promotes a loss of responsiveness of luteinizing hormone receptor in MA10 cells (28). These results suggest a role for Src-like tyrosine kinases in the regulation of G protein-coupled receptor signaling and desensitization.

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In this context, we have investigated the possibility that GRK2 could be regulated by Src-mediated tyrosine phosphorylation. We find that GRK2 is a new substrate for c-Src and, what is more important, that agonist stimulation of β2-adrenergic receptors leads to rapid phosphorylation of GRK2 on tyrosine residues, which results in an enhancement of GRK2 intrinsic activity. Our results indicate that tyrosine phosphorylation of GRK2 is inherent to GPCR activation, thus suggesting an important role in the modulation of GRK2 cellular functions.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine GRK2 was overexpressed and purified from baculovirus-infected SF9 cells as described (29). Purity of the GRK2 preparation as determined by SDS-polyacrylamide gel electrophoresis was >95%. Recombinant baculovirus for GRK2 was kindly provided by Dr. J. L. Benovic at the Thomas Jefferson Cancer Institute of Philadelphia. The cDNAs encoding human p60(src) and the constitutively active Y527F p60(src) were provided by Dr. S. Gutkind (NIH, Bethesda, MD). COS-7 cells, HEK-293 cells, and human Jurkat T lymphocytes were from the American Type Culture Collection (Manassas, VA). Culture media and LipofectAMINE were from Life Technologies, Inc. Protein A-Sepharose, isoproteorin, and heparin (M, 6,000) were obtained from Sigma. [γ-32P]ATP was purchased from Amersham Pharmacia Biotech, and [35S]methionine- and [35S]cysteine-labeling mixture was from NEN Life Science Products. Purified pp60src was obtained from Upstate Biotechnology. All other reagents were of the highest grade commercially available.

**Cell Culture and Transfection**—COS-7 and HEK-293 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum at 37 °C in a humidified 7% CO2 atmosphere. Transfections were performed on 70% confluent monolayers in 100-mm dishes for immunoprecipitation assays or 60-mm dishes for 35S-labeling experiments. For transient transfection, cells were incubated at 37 °C in serum-free OPTIMEM (5 ml containing 5 μg of DNA100-mm dish or 3 μg/60-mm dish) plus 8 μl of LipofectAMINE reagent. After gentle rocking for 90 min at 4 °C, the lysates were clarified by centrifugation, and an aliquot (30 μl of a 50% suspension of an agarose-starved for 90 min before the experiment as reported (31). Treatments with the Src inhibitor PP2 (5 μM, Calbiochem) were initiated 15 min before isoproteorin stimulation. For metabolic labeling, transiently transfected HEK-293 cells were kept for 2 h in methionine and cysteine-free DMEM and then incubated for 15 min in DMEM supplemented with 250 μCi/ml [35S]methionine- and [35S]cysteine-labeling mixture as reported (32). Labeled cells were used for antiphosphoserine immunoprecipitation experiments as described below. After SDS-polyacrylamide gel electrophoresis (PAGE), the presence of GRK2 was subsequently analyzed by 35S detection or immunoblot.

**Immunoprecipitation and Western Blotting**—For immunoprecipitation, the cells were washed twice with ice-cold phosphate-buffered saline supplemented with 1 mM sodium orthovanadate, solubilized in 700 μl/100-mm dish or 400 μl/60-mm dish of RIPA buffer (50 mM Tris, pH 7.5, 0.3 μM NaCl, 1% (w/v) sodium deoxycholate, 1% (v/v) TX-100, 0.1% SDS, 1 mM sodium orthovanadate, plus a mixture of protease inhibitors). After gentle rocking for 90 min at 4 °C, the lysates were clarified by centrifugation, and an aliquot (30 μl) was used to assess protein overexpression. Before GRK2 immunoprecipitation, the supernatants were preclared by overnight incubation with a preimmune serum plus 0.5 mg/ml bovine serum albumin. After incubation at 4 °C with Protein A-Sepharose for 1 h and centrifugation, the beads (preimmune controls) were washed with ice-cold RIPA buffer and incubated with the phosphorylation buffer to remove the detergent, and casein or rhodopsin were added to a final concentration of 30 μg or 1 μg, respectively. The phosphorylation reaction was initiated by adding 20 μl of kinase reaction buffer (30 mM Tris-HCl, pH 7.2, 8 mM MgCl2, 1.4 mM EDTA, 1 mM EGTA, 4.5 mM NaF, 0.25 mM Na3VO4, 160 μM ATP, and 5,000 cpm/pmol [γ-32P]ATP). After incubation for 30–60 min at 30 °C, the reaction was stopped with SDS sample buffer, and the phosphorylated proteins were resolved by SDS-PAGE, revealed by autoradiography, and quantitated as reported (29, 33). For the in vitro phosphorylation studies, recombinant GRK2 protein at the concentrations depicted in the figure legends was incubated with purified c-Src kinase (9.2 nM, Upstate Biotechnology) in a final volume of 15–30 μl of kinase reaction buffer (100 mM Tris-HCl, pH 7.2, 25 mM MgCl2, 2 mM EDTA, 0.25 mM Na3VO4, 125 mM magnesium acetate, 50 μM ATP, and 2,000 cpm/pmol [γ-32P]ATP) in the presence or absence of 0.25 mM heparin to inhibit GRK2 autophosphorylation (34). After the indicated time at 30 °C, the reaction was stopped by the addition of 2× SDS-PAGE sample buffer. Phosphorylated proteins were resolved by electrophoresis in 8% polyacrylamide gels and visualized by autoradiography. Enolase phosphorylation assays were performed as described (35).

**RESULTS AND DISCUSSION**

To explore the possibility that GRK2 could be a target for Src-like tyrosine kinases, recombinant GRK2 was incubated with purified c-Src under phosphorylating conditions. The presence of Src promoted a marked increase in GRK2 phosphorylation (Fig. 1A). The phosphorylation of GRK2 is likely due to the activity of Src, since these initial experiments were performed in the presence of heparin, a well known inhibitor of GRK2 activity and autophosphorylation (34). Control experiments indicated that heparin had no effect on Src intrinsic activity toward other substrates such as enolase (Fig. 1B). To further confirm that GRK2 was directly phosphorylated by Src on tyrosine residues, we performed similar phosphorylation experiments followed by immunoblot analysis using antiphosphotyrosine antibodies. Fig. 1C shows that these antibodies only recognized GRK2 after previous incubation with Src. These results indicate that GRK2 is a substrate for Src tyrosine kinase in vitro.

To explore if phosphorylation of GRK2 by Src could have a physiological meaning, we sought to determine the kinetic parameters of the phosphorylation reaction. Since results similar to those shown in Fig. 1, A and C, were obtained in the absence of heparin (data not shown), we routinely performed the experiments in the latter conditions using basal GRK2 autophosphorylation controls to assess Src-mediated GRK2 phosphorylation in each experimental determination. Fig. 2A indicates that GRK2 is readily phosphorylated by c-Src reaching a plateau at 15–20 min of incubation. The stoichiometry of phosphorylation of 10 nM GRK2 induced by equimolar Src in the absence of heparin was 0.86 ± 0.08 mol of phosphate incorporated/mol of GRK2 (mean ± S.E. of 4 independent experiments). This value is very close to that reported for PKC phosphorylation of GRK2 (0.5–0.9 mol P/mol of GRK2; see Refs. 15 and 16) and suggests that GRK2 is phosphorylated by c-Src on a tyrosine residue.

Double-reciprocal plot analysis (Fig. 2B) reveals that c-Src displays an apparent K_m for GRK2 of ~12 nM, with a V_max of 1.3 nmol/min/mg of protein. This K_m is lower than that reported (36–38) for well known Src substrates such as fodrin (0.3 μM), tubulin (2 μM), gelsolin (6 μM), or Shc (90 nM). Therefore, our data indicate that GRK2 is a high affinity substrate for c-Src.
GRK2 Phosphorylation by Src

**Fig. 1.** GRK2 is phosphorylated by Src tyrosine kinase in vitro. A, increased GRK2 phosphorylation in the presence of c-Src. Samples containing recombinant GRK2 (60 nM) alone (autophosphorylation control) or in the presence of heparin (0.25 ng/ml) and purified pp60src (9.2 nM) as indicated were incubated under phosphorylation conditions for 30 min as described under “Experimental Procedures,” followed by SDS-PAGE and autoradiography. The arrows indicate the bands corresponding to the migration of GRK2 and Src. B, heparin had no effect on enolase phosphorylation by Src. Samples containing pretreated enolase substrate (0.83 µg/ml) were incubated with purified Src (9 nM) under phosphorylation conditions with or without heparin (0.25 µg/ml). Enolase and pp60src bands revealed by autoradiography are indicated. C, GRK2 is phosphorylated on tyrosine residues. Recombinant GRK2 (10 nM) and Src (9.2 nM) were incubated as in panel A. After SDS-PAGE, the samples were transferred to nitrocellulose filters, and phosphorylated proteins were detected by incubation with a 1:6,000 dilution of PROPHOSPHOTYROSINE antibody (anti-pTyr BLOT). The migration of GRK2 and pp60src are indicated on the left, and the Mₐ of resolved protein standards are shown in kilodaltons on the right.

for c-Src in vitro and suggest that tyrosine phosphorylation of GRK2 would also take place in the cellular environment.

To determine whether GRK2 could be phosphorylated by Src in vivo, we coexpressed this kinase with wild type Src or the constitutively active Y527F Src mutant (39) in COS-7 cells. The pattern of phosphorylated proteins in cell lysates was investigated using antiphosphotyrosine antibodies. As shown in the left panel of Fig. 3A, a protein that comigrates with GRK2 is markedly tyrosine-phosphorylated only in cells transfected with constitutively active Src. Similar levels of GRK2 (upper right panel) and Src (lower right panel) overexpression were obtained in the different conditions, as assessed by reprobing the blots with specific antibodies. In addition, antiphosphotyrosine immunoblot analysis of GRK2 immunoprecipitated from cells transiently co-transfected with the kinase and Y527F Src demonstrate that GRK2 is indeed phosphorylated on tyrosine residues under these conditions (Fig. 3B). The titration experiment shown in Fig. 3B indicates a correlation between the phosphotyrosine content of immunoprecipitated GRK2 and an increased expression of the Src mutant, suggesting a direct relationship between the levels of Src activity and tyrosine phosphorylation of the GRK2 cellular pool. We also performed similar experiments in HEK-293 cells. Our data show that GRK2 could be immunoprecipitated with an agarose-conjugated phosphotyrosine antibody only after coexpression with the Src mutant (Fig. 3C and data not shown). GRK2 protein present in the phosphotyrosine immunoprecipitates was detected either by Western blot using a GRK2 antibody or by 35S-radiolabeling and autoradiography. It is worth noting that a tyrosine-phosphorylated band with a slightly slower migration than GRK2 was detected by the kinase antibody (Fig. 3C, upper panel) but not by 35S-labeling (Fig. 3C, lower panel). Such a band is also present in some experiments in GRK2 immunoprecipitates analyzed with phosphotyrosine antibodies (Fig. 4A and data not shown). It is tempting to suggest that such a band represents a minor cellular pool of another form of tyrosine-phosphorylated GRK2, which deserves to be characterized in future experiments.

Taken together, our results indicate that GRK2 is phosphorylated by activated Src tyrosine kinase in living cells. We next tested the hypothesis that GPCR activation could be the physiological trigger of GRK2 phosphorylation on tyrosine residues. Several recent reports have established that stimulation of β-adrenergic and other GPCR leads to c-Src-mediated phosphorylation of several cellular proteins. Phosphorylation of adapter proteins such as Shc and Gab1 is followed by Ras-dependent modulation of the MAPK cascade (reviewed in Refs. 7 and 18). Activation of β₂-adrenergic receptors also leads to Src-medi-
Increased Src activity leads to GRK2 phosphorylation on tyrosine residues in COS-7 cells. A, COS-7 cells were transiently transfected with GRK2 alone (lane 1) or in combination with wild type c-Src (lane 2) or the constitutively active c-Src mutant Y527F (lane 3). Cell lysates were resolved by SDS-PAGE, blotted, and analyzed by a monoclonal antiphosphotyrosine antibody (left panel). After stripping of the blots, GRK2 and Src expression was determined by using anti-GRK2 (upper right panel) or anti-Src antibodies (lower right panel), respectively. The arrows on the left indicate the migration of GRK2 and Src, determined by running an aliquot of recombinant GRK2 protein and purified Src in the same SDS-PAGE gel (Std lane) as a control. Molecular weight standards in kilodaltons are also indicated. B, the indicated quantities of cDNA of the constitutively active c-Src mutant Y527F were transiently cotransfected in COS-7 cells with a given amount (1.5 μg) of GRK2 cDNA. RIPA buffer lysates were immunoprecipitated (IPP) with a preimmune serum or with the anti-GRK2 polyclonal antibody Ab-FP1. After SDS-PAGE, the samples were analyzed by immunoblot using an anti-phosphotyrosine antibody (upper panel). After stripping, the same blots were developed with anti-GRK2 antibodies (lower panel). The migration of recombinant GRK2 resolved in the same gel is indicated (Std lane). Data in panels A and B are representative of five independent experiments. C, GRK2 can be detected in antiphosphotyrosine immunoprecipitats (anti-pTyr IPP) of GRK2 from RIPA buffer lysates were resolved by 8% SDS-PAGE, blotted, and analyzed with antiphosphotyrosine (anti-pTyr) antibodies (anti-pTyr IPP). After stripping of the blots, the presence of GRK2 was analyzed in the same gel using a specific GRK2 antibody (anti-GRK2 IPP). Nonstimulated controls were taken as the basal condition. Each data point represents the mean ± S.E. from at least three independent experiments. D, isoproterenol-stimulated tyrosine phosphorylation of GRK2. Tyrosine phosphorylation of GRK2 was measured by scanner laser densitometry, and the data were normalized to the amount of GRK2 protein present in the immunoprecipitates, as assessed with the GRK2 antibodies. Nonstimulated controls were taken as the basal condition. Each data point represents the mean ± S.E. from at least three independent experiments. E, isoproterenol-stimulated tyrosine phosphorylation of GRK2 was assessed by isoproterenol-stimulated tyrosine phosphorylation of GRK2. Tyrosine phosphorylation of GRK2 was measured by scanner laser densitometry, and the data were normalized to the amount of GRK2 protein present in the immunoprecipitates, as assessed with the GRK2 antibodies. Nonstimulated controls were taken as the basal condition. Each data point represents the mean ± S.E. from at least three independent experiments.
The use of transient transfection experimental systems raises the question of whether GRK2 phosphorylation on tyrosine residues could also be observed in more physiological conditions. In this regard, preliminary data in our laboratory indicate that tyrosine phosphorylation of endogenous GRK2 can be detected in Jurkat cells and that its tyrosine phosphorylation levels are modulated upon stimulation with agents that activate tyrosine kinases of the Src family or with CXCR4 receptor agonists (data not shown).

To determine whether tyrosine phosphorylation of GRK2 affected its function, we compared the activity of GRK2 in cells expressing this kinase in the absence or presence of the constitutively active Y527F Src mutant. Y527F Src expression promotes an increased level of GRK2 tyrosine phosphorylation that can be detected both in cell lysates (Fig. 5A) or after immunoprecipitation with specific GRK2 antibodies (Fig. 5B). When immunoprecipitates containing similar quantities of GRK2 (Fig. 5B, lower panel) were tested for the ability of GRK2 to phosphorylate soluble GRK2 substrates, a clear increase in GRK2 activity was detected in the tyrosine-phosphorylated samples (between a 1.4- and 2.5-fold increase in activity toward casein, Fig. 5C). Similar results are obtained when a GPCR such as rhodopsin is used as a substrate (1.7 ± 0.29-fold increase in phosphorylation, Fig. 5D). The range of this stimulation was markedly reduced (Fig. 4C), thus suggesting that this process is in fact due to the activity of Src.

Taken together, our data indicate that GRK2 is a substrate for c-Src in vitro and in living cells and that stimulation of the β2-adrenergic receptor leads to rapid tyrosine phosphorylation of GRK2 and modulation of GRK2 function. Since activation of β2-adrenergic receptor has been shown to increase Src-mediated protein phosphorylation (18–21) and we report that GRK2 is a good substrate for c-Src in vitro, our results strongly suggest that this tyrosine kinase is directly involved in the observed agonist-induced tyrosine phosphorylation of GRK2, although the participation of other tyrosine kinases cannot be ruled out. Higher GRK2 functionality upon phosphorylation by c-Src would favor a diminished response of GPCR to extracellular messengers. Consistently, the level of activity of Src kinase has been reported to correlate with a loss of responsiveness of luteinizing hormone receptors in MA10 Leydig cells (28) or of α2- and β-adrenergic receptors in pinealocytes (41). However, different effects have been observed in other systems (26, 27), thus suggesting that the overall effect of c-Src would depend on the combination of its actions at different levels of the GPCR-mediated signaling pathway by phosphorylating targets such as α2c, cAMP phosphodiesterase (41), or GRK2.

The fact that GRK2 is one of the targets of agonist-activated Src is consistent with the reported presence of GRK2 as part of the multicomponent complexes formed upon GPCR stimulation (30, 42, 43). The rapid kinetics of agonist-induced phosphorylation of GRK2 on tyrosine residues is similar to that observed for other cellular targets of GPCR-dependent Src activity, such as dynamin, Gαi1, or Shc (20, 21, 40, 44), indicating that agonist-induced GRK2 phosphorylation is an early event in GPCR signal transduction. On the other hand, its transient nature suggests the existence of active GRK2 dephosphorylation mechanisms, which may contribute to the explanation of why GRK2 phosphorylation on tyrosine residues has not been previously detected. Moreover, our results are of particular interest in the context of recent data published during the preparation of this manuscript, indicating that arrestin proteins can act as adaptor molecules for recruiting Src to activated GPCR (23). Thus, the following sequence of events can be envisaged. Agonist-occupied receptors are rapidly phosphorylated by GRK2; β-arrestin then binds to the phosphorylated GPCR, allowing Src recruitment and activation (23) so the subsequent phosphorylation on tyrosine residues of GRK2 and other adaptor and signaling proteins can take place. Src-dependent phosphorylation of GRK2 as inherent to GPCR signaling may provide a positive feedback loop mechanism (45); increased GRK2 activity would help to block G protein-mediated signaling while reinforcing the arrestin/Src/Ras/MAPK pathway. This suggested function of GRK2 is in line with the previously proposed role for GRKs and arrestins in “signal switching” (7) and may also have physiological implications. In this regard, GRK2 appears to be important in the function and disfunction of the cardiovascular system (reviewed in Refs. 1, 5, and 6). Recent data indicate that c-Src activation plays a critical role in hypertrophic growth regulation of cardiac myocytes (Refs. 46 and 47 and references therein). Therefore, the interaction between Src and GRK2 in such a pathway may help to understand the physiological consequences of the lack of GRK2.
activity during embryonic development (lethal phenotype with marked myocardial hypoplasia (48)) or of the increased levels of GRK2 in congestive heart failure and cardiac hypertrophy situations (49–51).

Finally, it is tempting to suggest that tyrosine phosphorylation of GRK2 may also modulate other unknown functions of this kinase in the GPCR multimolecular signaling complex or affect other aspects of GRK2 cellular functions and regulation, which we are currently investigating. On the other hand, our data also put forward GRK2 as a potential target for cross-talk and their interaction with other cellular proteins.

Acknowledgments—We thank Drs. J. L Benovic and J. S. Gutkind for experimental tools and Dr. Ana Ruiz-Gómez for generously providing purified recombinant GRK2. Drs. P. Penela, A. Ruiz-Gómez, C. Ribas, and A. Aragay are acknowledged for critical reading of the manuscript. We also thank A. Morales for secretarial assistance and Professor F. Mayor for continuous encouragement.

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